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# Studies on the Artificial Sex Control in Rainbow Trout

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NIJIMASU NO JIN'ITEKI SEITOGYO NI KANSURU KENKYU (STUDIES ON THE ARTIFICIAL SEX CONTROL IN RAINBOW TROUT, Salmo gairdneri) p. 1

Houji OKADA (Hokkaido Fish Hatchery)

Hokkaidoritsu Suisan Fukajo Kenkyu Hokoku (Scientific Reports of the Hokkaido Fish Hatchery), No. 40. December 1985.

## Abstract

**Abstract**

Since Yamamoto (1953, 1958) first reported functional sex reversal in medaka (*Oryzias latipes*) by administration of sex homones, artificial sex reversal was accomplished in several kinds of fresh water fishes such as in gold fish (Yamamoto and Kajishima, 1968), Tilapia (Clemens and Inslee, 1968), and guppy (Takahashi, 1975a). The results obtained in these studies provide not only information about the process of sex differentiation and genetic bases of male heterogamety (XY), but also suggest the possibilities of practical applications of sex control on fish culture. The present study was carried out to accomplish functional sex reversal by sex steroids and the production of triploids by hydrostatic pressure. The musculinization of genetic females by androgen treatment was tried to get XX male which sire all female progenies. The feminization of genetic males by the estrogen treatment was tried to get YY males which sire all male progenies. The production of all female triploids was also tried to get sterile fish. The results obtained in this study are summarized as follows;

1) Some genetic females had a gonad showing signs of meiosis at the swim-up stage. Number of oocytes increased at 4 weeks after the swim-up and most of the germ cells changed into oocytes at 6 weeks after the swim-up.

2) Dietary 17$\alpha$-methyltestosterone (0.8 and 5 ppm in food) effected the musculinization when the oral treatment started within 2 weeks after the swim-up.The treatment which was started 4 weeks or later had no effect on sex defferentiation. Long-term treatment (8 weeks) started within 2 weeks after the swim-up with the same doses having a strong effect on the musculinization.

3) The most effective dose of 17 $\alpha$-methyltestosterone and 11-ketotestosterone were 0.5-1.0 ppm and 5 ppm, respectively. An excess dosage of androgen had no effect on musculinization.

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4) The androgen induced XX males sired all female progenies in the $F_1$ generation when mated with normal females. All females produced were normal in reproductive function and produced a 1 : 1 sex ratio in $F_2$ generation. The results of these experiments demonstrate that rainbow trout is female homogamety (XX) and functional sex reversed XX males produce all female progenies with normal reproductive function.

5) The gonads of genetic males did not show any signs of meiotic activity at the swim-up stage. The testes were identified by the development of seminiferous tubules and the formation of spermatogonial cysts of gonia at 25 weeks after the swim-up, but meiotic activity was not observed.

6) The oral treatment of estradiol-17$\beta$ (50 ppm and 100 ppm) from the first feeding had an effect on the feminization of genetic males, but the ratio of sex reversal was low.

7) The estrogen induced XY females sired progenies of which the sex ratio of male to female was 3.0 in the $F_1$ generation. YY males in $F_1$ were detected by progeny test and they sired all male progenies in the $F_2$ generation. All produced males were normal in reproductive function and produced a 1 : 1 sex ratio in the $F_3$ generation. The results of these experiments demonstrate that rainbow trout is male heterogamety (XY) and induced YY male produces all male progenies with normal reproductive function.

8) The triploidy was induced by hydrostatic pressure (650 kg/cm², 6 min.). All triploid males matured up to 37 months age after hatching. Matured males repesented secondary sex characteristics and produced aneuploid sperm. On the other hand, all of triploid females did not mature. Their germ cells remained in the oogonial stage and were immature until 37 months age.

9) The production of sterile stocks was tried by all female triploidy. After fertilization with hormonal induced XX male with normal females, fertilized eggs were pressed at 650 kg/cm² hydropressure for 6 minutes. Eighty-six percent of the treated fry were triploid females and the others were diploid females.

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## Introduction

Rainbow trout were first transplanted to Japan in 1877 (10th year of Meiji) from the U.S.A., the place of origin (Inaba, 1968). Since then cultivation and propagation have been tried in many parts of Japan. Breeding and brood-stock production are comparatively easy, thus this is one of a few examples of successful transplantations of foreign species. The national production was about 100 tons in early Showa (about 1930's). The maximum production before World War II was 516 tons in 1943. Before World War II, production from both release in lakes and cultivation in ponds was not great. However, due to the export of frozen fish to the U.S. which began in 1951, the production of

cultivated rainbow trout has increased greatly. The advancement of freezing technology has enabled the export of pan-size fish to the U.S. In 1964, the production reached 5,400 tons and 36 % of them, about 2,000 tons, were exported.

Rainbow trout cultivation has expanded. However, the export to the U.S. became difficult due to the trend to a strong yen in late 1965 and the cultivation industry was thrown into disorder. The industry has continued to make efforts in improving the breeding techniques, in saving energy and in expanding the market. In recent years, production is 16,000-20,000 tons. However, added to the rise in the cost of feed, the consumption of small-sized fish for caramelized and broiled fish which are the main constituents of the demand has reached a ceiling and the production has remained on the same level for the past few years. In order to reduce production cost and to improve production efficiency, research has been done in the improvement of broodstocks and breeding techniques and in the establishment of measures to counter fish diseases. In order to extend the demand and to increase the additional values, it is thought that the production of large-sized fish of 1-2 kg and a guarantee of market for fresh fish are necessary.

In the production of large-sized fish, the following problem areas have been noted: a long period of cultivation, growth suspension due to sexual maturation and a deterioration or a decrease in commodity values. These

difficulties are raising production cost and making the production of large-sized fish problematic. In order to enlarge the demand for production of large-sized fish, it is necessary to solve these problems caused by maturation.

The age of sexual maturation of rainbow trout is different for males and females. Males mature in 1-2 years (12-24 months after hatching) and females in 2-3 years (24-36 months after hatching). Males mature one year earlier than females. Therefore most of the problems caused by maturation of the first- and the second-year fish are due to early maturation of males. If all-female stocks can be realized by controlling the sex ratio, the production cost of large-sized fish can be reduced. Furthermore the sterilization of broodstocks prevents completely the difficulties arising from maturation and enables the production of large-sized rainbow trout. The all-female or sterilized stocks do not have the growth suspension due to maturation, therefore, the cultivation period can be shortened. The all-female stocks are advantageous not only to the production of large-sized fish but also to that of breeding eggs. In the artificial fertilization of rainbow trout in which only a few males are required in ratio to females, the rise in the female ratio directly influences the production of breeding eggs.

p. 4

In the production of breeding eggs for large-sized fish, females are favorable. The difference in growth in

males and females appears several months before maturation. It is known that mature fish are larger and more active than immature fish in salmonids. In the production of small-sized fish, males can be favorable. In the cultivation of masu salmon (young salmon), the difference in males and females due to phase differentiation is great. Smolting of females reduces the commodity value as young salmon, therefore, it is thought that males are advantageous in the cultivation of young salmon. Thus advantages and disadvantages of males and females are observed according to the form of production depending on different species or even among the same species. The production efficiency can be improved if the sex ratio in broodstocks advantageous to each form of cultivation can be established.

The sex ratio control and the sterilization of broodstocks are useful for the cultivation of salmonids, especially that of rainbow trout. They are also effective in ensuring the release stock for propagation. Especially in the masu salmon propagation industry in which the release stocks are produced by pond breeding, the increase in the female ratio directly affects the increase in the stock production and the smolting ratio which in turn enables the effective management of the industry.

The sex ratio control in Osteichthyes was reported for the first time by Yamamoto (1953, 1958). He reported in his study that the feminization of genetic males and

masculinization of genetic females could be achieved in medaka (Oryzias latipes) and that all females or all males could be obtained in $F_1$ and $F_2$ generations by mating the sex-reversed fish with normal opposite sexes. Later the same results have been reported for gold fish (Yamamoto and Kajishima, 1968), guppy (Takahashi, 1975a, b) and Tilapia (Clemens and Inslee, 1968). The experiments on medaka and gold fish by Yamamoto observed the reversal of sex differentiation by sex steroids and the $F_1$ sex ratio as an experimental method for elucidation of the mechanism of sex differentiation and the sex chromosomes. The results show that the method used is a very useful one not only as a simple biological experimental method but also as a technique of controlling the sex ratio in the cultivation and propagation of fishes.

In salmonids, many researches have been done due to the fact that the male/female ratio necessary for the artificial fertilization is not 1 and that the difference in advantages and disadvantages is great between males and females. The fluctuation in the sex ratio of the treated generation by sex steroids has been reported for rainbow trout (Okada, 1973; Johnstone et al., 1978), coho salmon (Goetz et al., 1979) and masu salmon (Nakamura, 1981a). Furthermore researches on rainbow trout (Okada et al., 1979; Johnstone et al., 1979), coho salmon (Hunter et al., 1982) and Atlantic salmon (Johnstone and Youngson, 1984) have reported that these species of fish

are of XX-XY type from the experimental results of the reversal of functional sex differentiation by sex steroids and from the sex ratio of the $F_1$ generation. It was confirmed that all-female $F_1$ could be obtained from XX males induced by androgen.

The induction of sex differentiation by sex steroids has been reported for many species of fish. Takahashi (1978) discussed the difference in the mechanism of sex differentiation between fishes and Amphibia and the sex differentiation inducers. He also stated the principles of treatment methods for the sex reversal by sex hormone treatment. Recently, Yamazaki (1982) and Yamazaki (1983) have divided the sexes of fish largely into the physiological sex and the genetic sex and discussed the methods of controlling each sex and their applications to the fish culture. In the present study, we discussed in detail the administration time and dosage level for masculinization by androgen using genetic females of rainbow trout alone as sample materials. The most suitable administration method was determined and the maturation of all females produced by XX males and the sex ratio of the $F_2$ generation were examined. Results showed that the all females were functionally and genetically normal females. For feminization, genetic males alone were used as sample materials and the dosage level of estrogen was studied. Furthermore YY males included in males produced by XY females were confirmed in $F_1$ of sex ratio of 1:3 female to male.

Maturation of all males which were produced by YY males and the sex ratio of the $F_3$ generation demonstrated that the all males were functionally and genetically normal males. All-female triploids were produced using the polyploidy method for chromosomes by addition of hydrostatic pressure and the sex ratio control method reported recently by Onosato (1983a). Sterilization of broodstocks were also discussed.

In this study, the following are discussed with regard to rainbow trout. (1) Sex chromosomes, (2) reversal methods of sex differentiation by sex steroids, (3) production of all females in the $F_1$ generation or of all males in the $F_2$ generation from the sex-reversed fish and (4) sterilization by all-female triploids. Results are given below. p. 5

## Chapter I History of studies on artificial sex control in Osteichthyes

The artificial induction of sex differentiation in Osteichthyes was reported for the first time by Yamamoto (1953) using medaka (Oryzias latipes). He reported that genetic males (XY) could be reversed to functional females by oral administration of estrogen using d-rR in which the sex chromosomes could be identified by body colors. They were bred by mating white with red medaka. In the $F_1$ generation of sex-reversed XY females and normal XY males, YY males existed although their ratio was lower than the theoretical value. It was confirmed that YY males produced an all-male $F_2$ generation (Yamamoto, 1955). It was reported that the

genetic females (XX) were masculinized by oral administration of androgen and produced an all-female $F_1$ generation by mating with normal XX females (Yamamoto, 1958). In these reports, Yamamoto demonstrated a possibility of reversal of the genetic sex to the opposite sex by oral administration of sex steroids during the period from the stage before the sex differentiation to the one after it is completed. The same experiment described as above was carried out using gold fish, Carassius auratus. The following were confirmed: the functional masculinization by androgen, the production of all-female F1 by XX males, the functional feminization by estrogen, the production of an $F_1$ of a sex ratio of 1:3 female to male and the production of all males by YY males. It was also reported that the sex chromosomes were of XX-XY type (Yamamoto and Kajishima, 1968).

Takahashi (1975a, b) reported the masculinization of genetic females and the production of an all-female $F_1$ by XX males in guppy, Poecilia reticulata.

The artificial reversal of sex differentiation and the fact that sex-reversed fish produce an all-female $F_1$ or an all-male $F_2$ which were demonstrated with these species of fish were not only limited to scholastic interests such as the mechanism of sex differentiation and the elucidation of sex chromosomes but also were studied for the application to Tilapia and salmonids as a method of controlling the sex ratio in broodstocks.

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Clemens and Inslee (1968) reported the artificial masculinization and the production of an all-female $F_1$ using Tilapia, Tilapia mossambica and also demonstrated that the sex chromosomes were of XX-XY type. Nakamura and Takahashi (1973) studied the artificial feminization with the same species. Nakamura (1981b) made observations with the same species on the effects of time and duration of administration of sex steroids on sex differentiation with regard to artificial masculinization. He also discussed correct methods of administration.

As for salmonids, Ashby (1957) made observations on the effects of several sex steroids on the gonad of brown trout, Salmo trutta but no reversal of sex differentiation was observed. Yamazaki (1976) made observations on the effects of sex steroids on growth, maturation, reproductive activity, sex reversal and sterility in order to study the application of sex steroids in fish culture. He reported a rise in the male ratio by oral administration of 17$\alpha$-methyltestosterone in rainbow trout, Salmo gairdneri, and suggested the masculinization of genetic females.

Okada(1973) reported a rise in the female ratio by oral administration of estrone in rainbow trout and suggested the feminization of genetic males.

Johnstone et al.(1979, 1984) also reported the feminization by 17$\beta$-estradiol and the masculinization by 17$\alpha$-methyltestosterone in rainbow trout and in Atlantic salmon,

Salmo Salar. Furthermore Goetz et al.(1979) reported artificial feminization and masculinization by immersion treatment and continuous oral administration of these steroids in coho salmon, Oncorhynchus kisutch.

Nakamura(1981a) reported that feminization was possible by immersion treatment with 17$\beta$-estoradiol alone in masu salmon, Oncorhynchus masou, and discussed sex differentiation and the time of administration. After the 1970's, there have been many reports on the reversal of sex differentiation in salmonids by oral administration of sex steroids by combination of immersion treatment and oral administration or by immersion treatment alone. They demonstrated that by administration of appropriate sex steroids at the time of sex differentiation, the reversal of sex differentiation was induced in the same way as with medaka, gold fish and Tilapia. These reports on salmonids have studied the morphological change of gonads and the reversal of sex differentiation from the fluctuation in the sex ratios. The sex-linked genic characteristics are not known for salmonids, therefore, the genetic sexes of sample fish are not known. If sample fish of known genetic sexes were used, detailed studies would be possible. p. 6

Okada et al.(1979) observed that in functional sex reversal, all-female $F_1$ could be produced by mating sex-reversed males(XX) with normal females in rainbow trout. They reported functional masculinization and that the sex

chromosomes were of XX-XY type. In the same year, Johnstone et al.(1979) reported functional masculinization and feminization in rainbow trout and that the sex chromosomes were of XX-XY type.

Since then, Hunter et al.(1972) observed the $F_1$ sex ratio of 1:3 female to male by mating the sex-reversed females with normal males in coho salmon and reported functional feminization and mascuclinization and that the sex chromosomes were of XX-XY type. Johnstone and Youngson(1984) reported functional masculinization in Atlantic salmon.

As stated above, functional masculinization and feminization in rainbow trout, functional feminization in coho salmon and functional masculinization in Atlantic salmon were confirmed. It was elucidated that these three species of fish have the XX-XY type of sex chromosome composition. However, various morphological abnormalities in the testes and the absence of spermiducts have been observed during the process of maturation in the sex-reversed males. Complete sex reversal of males has not been achieved. Detailed administration methods using all females produced by the sex-reversed males is necessary in order to study these problems.

With regard to the viability of YY males, Johnstone et al.(1979) stated a possibility of death of YY males as reported in medaka by Yamamoto(1955) from the observation made of the sex ratio in $F_1$ of sex-reversed females(XY) and that it was not a 1:3 female to male ratio. On the other hand,

Hunter et al.(1982) suggested the viability of YY males. Thus there are unclear aspects in the viability of YY in salmonids. In no species have YY males and all-male $F_2$ been confirmed.

Sterilization in salmonids has been studied using such methods as administration of sex steroids(Yamazaki, 1976; Donaldson and Hunter, 1982) and $\gamma$ -ray irradiation (Tashiro, 1972). However, complete sterilization has not been achieved and a reliable method of sterilization has not been established.

Sterilization by polyploidy of chromosomes is a very interesting possibility. Artificial polyploidy of fish was reported for the first time by Swarup(1956, 1959) in the three-spined stickleback, Gesterosteus aculeatus, using low-temperature shock. Since then, the productions of triploids by the same treatment have been reported in plaice, Pleuronectes platessa(Purdom, 1972), carp, Cyprinus carpio (Gervai et al., 1980), channel catfish, Ictalurus punctatus (Wolters et al., 1981) and Rhodeus ocellatus(Ueno and Arimoto, 1982).

Recently, the polyploidy of chromosomes has been reported by temperature shock or pressure treatment after fertilization in zebra fish, Brachydanio rerio(Streisinger et al., 1981), Atlantic salmon, Salmo salar(Benfey and Sutterlin, 1984a) and rainbow trout, Salmo gairdneri(Chourrout, 1984) and the production of clones by gynogenesis and a production

method of polyploids were established.

It was made clear that triploids were produced in many species of fish by low- or high-temperature treatment after fertilization and their sexual maturation was studied. In plaice(Purdom, 1972; Lincoln, 1981), triploid females are sterilized and males mature but the sperm are abnormal. Sterilization of females was also observed in carp(Gervai et al., 1980). On the other hand, in channel catfish, both males and females were sterilized. In Rhodeus ocellatus (Ueno and Arimoto, 1982), sterilization of males was observed.

Onosato(1983) observed a high rate of occurrence of polyploids in salmonids such as salmon, Oncorhynchus keta, masu salmon, O. masou, and rainbow trout by pressure treatment immediately after fertilization and reported that this treatment was more effective than temperature shock treatment.

There is no known study of fertility in artificial triploids in salmonids, however, a high rate of polyploidy has been reported in sterile brook trout, Salvelinus fontinalis, which appeared in a hatchery(Allen and Stanley, 1978). The same phenomenon was observed in rainbow trout and sterility in triploid females was suggested(Thorgaard and Gall, 1979). Fertility in triploids differs according to species or sex. However, it has been reported that females of plaice, carp and channel catfish were sterilized. p. 7

## Chapter II Masculinization of genetic females

In order to study artificial masculinization by sex steroids, the normal process of sex differentiation was observed using genetic females alone as samples. Effects of time and duration of the administration of sex steroids and the difference in the dosage level on sex differentiation were observed and effective treatment methods were discussed. In order to study the production of an all-female population using sex-reversed fish whose reversals were confirmed genetically, the maturation characteristics of sex-reversed fish, the sex ratio of $F_1$, the maturation characteristics of all females in $F_1$ and the sex ratio of $F_2$ were observed.

The application to rainbow trout breeding of all-female production by sex-reversed fish was studied.

### 1. Process of sex differentiation of genetic females

Histological observation was made in order to elucidate the normal process of sex differentiation of females.

#### 1) Sample fish and methods

All-female populations obtained by mating sex-reversed males(XX) with normal females were used as samples. Specimens were collected regularly from hatch-out to swim-up up to the 8th week. They were fixed in Bouin's solution. Paraffin sections were made by the standard method, stained with Delafield hematoxylin-eosin and examined under a microscope. The breeding water temperature for samples was 10.5°C.

2) Observation results

Development of the gonads at various stages are described below.

Hatch-out(total effective temperature 320°C): The gonads are observed in pairs beside the rudiment of the air-bladder directly below the kidney. The oogonium is already surrounded by the gonad epithelial cells and 1-2 of them are observed (Plate I, 1). The oogonium is round. The cell diameter is 10-17 μm and the nuclear diameter 10 μm. It is distinguished as a large and bright cell. Diameter of the gonad is about 20 μm but it becomes smaller at the end of the body and then disappears. The reproductive cells are not seen and the somatic cells alone are in the structure.

Swim-up (total effective temperature 590-610°C): Diameter of the gonad becomes 50 μm. A cavity which is thought to be the rudiment of the blood vessel is seen at the base. The oogonia increase in number. The cell diameter is reduced to 9-12 μm and the nuclear diameter to 7-8 μm. The pairing figure of chromosomes is seen on rare occasions and the transition to meiosis was recognized (Plate I, 2).

2-4 weeks after swim-up (total effective temperature 750-895°C): Diameter of the gonad increases to 50-100 μm. The blood circulatory system at the base becomes clear and the somatic cells multiply. The oogonia increase and the cyst membrane is formed. The nuclear diameter decreases to 5-8 μm. Only a few oogonia at the synapsis stage are seen

in the 2nd week but they constitute about 30 % in the 4th week.

6 weeks after swim-up (total effective temperature 1,041°): The gonad grows to a size of 200 x 100 μm. Most reproductive cells correspond to the oocytes of the karyosome stage or the early perinucledus stage (Plate I, 3).

8 weeks after swim-up (total effective temperature 1,190°C): The gonad grows to 300 x 200 μm. Many oocytes in the early perinucleolus stage of cell diameter 30-50 μm are observed. The ovary becomes clearer morphologically (Plate I, 4).

From the observations described above, it became clear that the transition from the oogonium to the oocyte of early meiosis begins in some reproductive cells at the time of swim-up. However, up to the 2nd week after swim-up, the active multiplication of the oogonium is observed and the transition to meiosis is rare. After the 4th week, the oocytes in the synapsis stage increase and grow to the early perinucleolus stage in the 6th week.

If we regard the transition from oogonia to oocytes as a histological sign of ovarian differentiation, the sex differentiation to females in rainbow trout begins already at the swim-up stage, however, its ratio is low in the 2nd week. Many oocytes differentiate after the 3rd week and sex differentiation is complete in the 6th week. p. 8

## 2. Time and duration of the administration of androgen and the masculinization ratio

In order to study the effects of androgen on sex differentiation, fluctuations in the sex ratio were observed through oral administration of sex steroids at various stages of growth from the swim-up stage.

### 1) Sample fish and methods

All females obtained by mating sex-reversed males (XX males) with normal females were used as samples. Androgen, 17$\alpha$-methyltestosterone(MT, Sigma Co.) was used. It was mixed with feed and administered orally. The feed mixture was made by spraying the feed with MT dissolved in ethanol which was air dried after spraying. The date of swim-up was set to be the date when half of the sample fish swam up. The period of the administration was established using this date as a basis. Sample fish were fed 3 times a day for 10 minutes. Except for the period of the administration, they were fed with ordinary assorted feed sold on the market. Breeding was continued up to the 24th-39th month after swim-up. Growth and sex ratio during that time were observed. Sex was determined mainly by crushing raw specimens and by examining them under a microscope. A fish with an ovary was determined as female, that with testes as male and that with both tissues as hermaphrodite. When the determination was difficult, a tissue section was made and then characterized.

Running water was used for the breeding of sample fish (water change rate, 2-3 times/h) and the water temperature was 10.5°C. The following 4 experiments were carried out in order to study the induction effects on the sex differentiation according to the different period of administration.

(Experiment I) MT treatment was started from the swim-up. 8 test groups were set up and the treatment was extended each week up to the maximum of 8 weeks. Effects of the extended period of treatment were observed (0-1 week - 0-8 weeks). The concentration of MT in the feed was 5 ppm.

(Experiment II) 8 test groups were set up and the start of treatment was delayed each week and the treatment ended on the 8th week after swim-up. Effects of the starting time of treatment were observed (0-8 weeks - 7-8 weeks). The concentration of MT in the feed was 5 ppm.

(Experiment III) 9 test groups were set up and the start of treatment was delayed each week and then the treatment was given for 8 weeks. A suitable period of treatment in the 8 weeks was observed (0-8 weeks - 8-16 weeks). The concentration of MT in the feed was 0.8 ppm.

(Experiment IV) 7 test groups were set up and MT was administered every 2 weeks at different periods after swim-up. Effective period of administration was observed (0-2 weeks - 12-14 weeks). The concentration of MT in the feed was 0.8 ppm.

Experiments I and II, III and IV were done simultaneously and a control group was set up in each experiment.

2) Results

**Table 1.** Body length at the time of start and termination of methyltestosterone (MT) treatment

| Treatment | BL±SD | |
|---|---|---|
| | Start | Termination |
| wk | mm | mm |
| 0-1 | 18.9±1.5 | 21.3±0.4 |
| 0-2 | 〃 | 22.4±0.9 |
| 0-3 | 〃 | 24.6±1.5 |
| 0-4 | 〃 | 26.7±3.1 |
| 0-5 | 〃 | 30.1±2.1 |
| 0-6 | 〃 | 30.8±3.4 |
| 0-7 | 〃 | 31.6±3.0 |
| 0-8 | 〃 | 29.5±2.0 |
| 0-8 | 18.2±1.9 | 36.0±3.8 |
| 1-8 | 20.1±3.3 | 35.8±2.9 |
| 2-8 | 22.7±1.0 | 37.9±2.7 |
| 3-8 | 25.9±0.9 | 36.2±1.3 |
| 4-8 | 27.1±2.3 | 35.3±3.8 |
| 5-8 | 28.7±1.5 | 37.1±2.8 |
| 6-8 | 31.6±2.3 | 36.6±2.1 |
| 7-8 | 34.3±2.3 | 36.9±2.6 |
| cont. | 18.7±1.6 (0wk) | 36.3±1.8 (8wk) |

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Body lengths at the time of start and termination of MT treatment in Experiments I and II are given in Table 1. In Experiment I, the body length of 18.9 mm at the time of start (swim-up time) became 21.3 mm at the end of the treatment in the group treated for 1 week (0-1 week). The body length became 29.5 mm in the group treated for the longest period (0-8 weeks). p. 9

In Experiment II, the MT treatment was started from a larger size according to the delaying time of start and the body length became 35-38 mm at the termination of 8 weeks.

No growth difference due to the time and the period of MT treatment was observed. No growth difference was observed between the treated groups and the control group which was not treated.

(2) Survival rate and effects of MT treatment on growth

**Table 2.** Mortality, survival rate and body length (BL) during 6 month-treatment with methyltestosterone after swin-up (Exp. I, II)

| Treatment | Mortality during treatment | 6 months after swim-up | |
|---|---|---|---|
| | | Survival rate | BL±SD |
| wk | % | % | mm |
| 0-1 | 0.0 | 71.7 | 79.2±7.0 |
| 0-2 | 3.1 | 87.3 | 75.2±6.8 |
| 0-3 | 3.9 | 82.0 | 74.1±7.8 |
| 0-4 | 2.4 | 47.3 | 84.0±7.5 |
| 0-5 | 3.9 | 85.9 | 71.1±8.1 |
| 0-6 | 2.4 | 90.7 | 75.1±8.8 |
| 0-7 | 3.9 | 89.8 | 77.8±7.7 |
| 0-8 | 3.9 | 89.8 | 78.2±6.1 |
| 0-8 | 5.0 | 82.5 | 78.0±8.8 |
| 1-8 | 17.0 | 69.5 | 94.1±8.2 |
| 2-8 | 8.0 | 69.0 | 92.3±8.5 |
| 3-8 | 9.0 | 86.0 | 85.9±8.8 |
| 4-8 | 7.0 | 46.5 | 80.3±7.2 |
| 5-8 | 6.0 | 86.0 | 86.5±8.2 |
| 6-8 | 9.0 | 23.5* | - |
| 7-8 | 6.0 | 86.0 | 81.0±8.0 |
| cont.(0-8) | 0.0 | 85.5 | 81.4±6.1 |

* Nearly all of the dead fish were accidentally lost by the trouble of the rearing apparatus.

In order to study the effect of MT treatment on survival and growth, the mortality, the survival rate 6 months after swim-up and the body lengths during the treatment in each group in Experiments I and II were examined. Results are given in Table 2.

Mortality during the treatment was 17.0 % in the group for which the treatment started one week after and ended after 8 weeks (1-8 weeks). In the other tests, mortality was low and no difference was observed due to difference in time and duration of treatment. Deaths in the 1-8 group were thought to be caused by something other than the MT treatment. Survival rate became low in the 0-4 and 4-8 groups in the 6th month, however, no difference due to MT treatment was observed. Decrease in the survival rate in the 6-8 group was caused by an accident in which the water supply was stopped. No growth difference due to MT treatment was observed. p. 10

(3) Masculinization ratio

The induction effect of methyltestosterone on sex differentiation was quantified through the masculinization ratio. The masculinization ratio was calculated by multiplying the number of appearance of males by 1 and that of hermaphrodites by 0.5 and then dividing by the total number of fish. The results are expressed as a percentage. Masculinization in Experiments I and II was examined on the 10th month and the 24th month. Results are given in Table 3. Results of Experiment III which were examined on the 10th month and the 24th month are given in Table 4 and those of Experiment IV on the 11th month and the 39th month are given in Table 5. Rates of appearance for females, males and hermaphrodites are given in Figs. 1-4.

In Experiment I for which the MT treatment was

**Table 3.** Masculinization ratio (MR.) of 5 ppm methyltestosterone treatment for various periods

| Treatment | 10 mo. after swin-up | | | | 24 mo. after swim-up | | | | M. R. |
|---|---|---|---|---|---|---|---|---|---|
| | F | H | M | BL±SD | F | H | M | BL±SD | |
| wk | | | | mm | | | | mm | % |
| 0-1 | 39 | | | 13.2±1.9 | 39 | 1 | 1 | 27.8±2.3 | 2.0 |
| 0-2 | 38 | 1 | 1 | 12.0±1.6 | 27 | | 1 | 15.5±2.7 | 3.7 |
| 0-3 | 40 | | 3 | 12.8±1.4 | 42 | 1 | 1 | 24.8±1.6 | 5.1 |
| 0-4 | 21 | | 4 | 13.1±1.6 | 21 | | 5 | 26.0±2.7 | 17.6 |
| 0-5 | 24 | 3 | 13 | 12.2±1.4 | 13 | 5 | 5 | 25.8±3.1 | 35.0 |
| 0-6 | 23 | 2 | 14 | 11.4±1.3 | 27 | 2 | 11 | 23.9±1.8 | 34.2 |
| 0-7 | 14 | 5 | 30 | 12.2±1.5 | 18 | 10 | 12 | 25.8±4.6 | 55.7 |
| 0-8 | 4 | 3 | 33 | 12.3±1.7 | 21 | 9 | 19 | 25.4±3.8 | 65.2 |
| 0-8 | 5 | | 27 | 13.4±1.1 | 1 | 6 | 31 | 27.4±4.9 | 87.2 |
| 1-8 | 13 | 3 | 24 | 14.6±1.2 | 5 | 2 | 3 | 26.3±3.0 | 59.0 |
| 2-8 | 11 | 2 | 17 | 13.9±1.6 | 6 | 7 | 9 | 28.6±2.5 | 58.7 |
| 3-8 | 26 | | 12 | 13.9±1.5 | 22 | 3 | 13 | 29.1±1.7 | 34.9 |
| 4-8 | 19 | | 6 | 14.8±1.2 | 10 | | 5 | 23.2±2.9 | 27.5 |
| 5-8 | 20 | | 11 | 13.7±1.4 | 23 | | 9 | 25.9±1.2 | 31.8 |
| 6-8 | 25 | | 3 | 14.0±0.9 | 5 | | 1 | 25.9±1.9 | 11.8 |
| 7-8 | 28 | | 7 | 12.7±1.5 | 23 | | 3 | 24.4±1.7 | 16.4 |
| cont. | 15 | | | 13.0±1.8 | 14 | | | 22.8±4.0 | 0.0 |

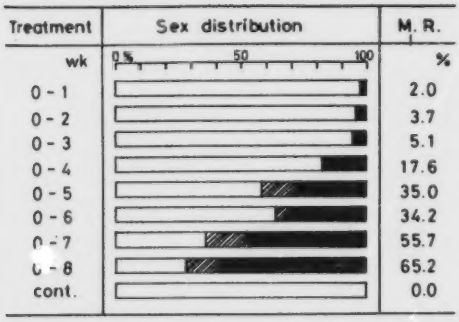


**Fig. 1.** Sex distribution of each test group treated with 5 ppm methyltestosterone during a week to 8 weeks from swin-up. Solid area and shaded area represent males and hermaphrodites respectively. In calculation the musculinization ratio (MR) male was evaluated as 1.0 and hermaphrodite was evaluated as 0.5.

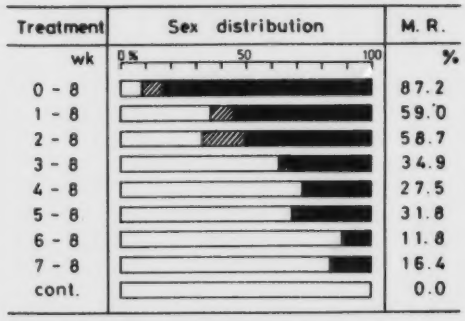


**Fig. 2.** Sex distribution of each test group treated with 5 ppm methyltestosterone from various time to 8th week after swim-up. Solid area and shaded area represent males and hermaphrodites respectively.

**Table 4.** Mascluinization ratio (M. R.) in 8-week methyltestosterone treatment (0.8 ppm) during various times of juvenile period

| Treatment | 10 mo. after swim-up | | | | 35 mo. after swim-up | | | | M. R. |
|---|---|---|---|---|---|---|---|---|---|
| | F | H | M | BL±SD | F | H | M | BL±SD | |
| wk | | | | cm | | | | cm | % |
| 0- 8 | 1 | 1 | 18 | 9.1±1.6 | | 1 | 14 | 20.7±2.1 | 94.3 |
| 1- 9 | | | 20 | 8.9±1.3 | | 2 | 6 | 21.3±1.6 | 96.4 |
| 2-10 | 2 | 1 | 16 | 8.7±1.4 | | 3 | 8 | 19.8±1.2 | 86.7 |
| 3-11 | 8 | 2 | 4 | 10.0±3.6 | 10 | 2 | 3 | 20.9±2.0 | 31.0 |
| 4-12 | 17 | | | 13.3±2.1* | 14 | | | 18.2±1.6 | 0.0 |
| 5-13 | 13 | | | 13.8±2.2* | 15 | | | 18.7±1.8 | 0.0 |
| 6-14 | 16 | | | 13.2±3.2* | 13 | | | 18.6±1.6 | 0.0 |
| 7-15 | | | | | 18 | | | 19.6±1.3 | 0.0 |
| 8-16 | | | | | 16 | | | 19.6±2.4 | 0.0 |
| cont. | 20 | | | 11.1±2.8 | 6 | | | 29.9±2.7 | 0.0 |

* 14 mo. after swim up.

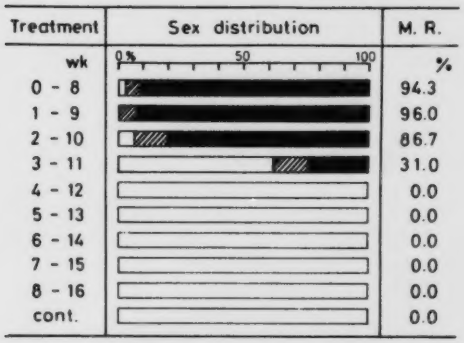


**Fig. 3.** Sex distribution of each test group treated with 0.8 ppm methyltestosterone during various 8 weeks. Solid area and shaded area represent males and hermaphrodites respectively.

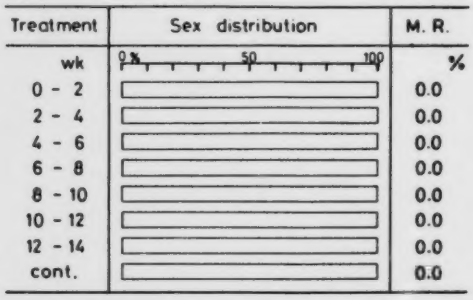


**Fig. 4.** Sex distribution of each test group treated with 0.8 ppm mthyltestosterone during various 2 weeks.

extended from swim-up, the masculinization ratio rose as the treatment period was extended. In Experiment II for which the start of the treatment was delayed, the masculinization ratio decreased as the start was delayed. However, even after one week of treatment, males appeared in the 0-1 group

**Table 5.** Masculinization ratio (M. R.) in 2-week methyltestosterone treatment (0.8 ppm) during various times of juvenile pemiod

| Treatment | 11 mo. after swim-up | | | | 39 mo. after swim-up | | | | M. R. |
|---|---|---|---|---|---|---|---|---|---|
| | F | H | M | BL±SD | F | H | M | BL±SD | |
| wk | | | | cm | | | | cm | % |
| 0-2 | 13 | | | 10.6±1.5 | 7 | | | 28.4±1.9 | 0 |
| 2-4 | 20 | | | 9.7±1.4 | 11 | | | 29.8±1.0 | 0 |
| 4-6 | 31 | | | 10.2±1.1 | 11 | | | 28.5±1.6 | 0 |
| 6-8 | 21 | | | 10.9±2.0 | 20 | | | 29.9±1.4 | 0 |
| 8-10 | 15 | | | 11.3±1.2 | 21 | | | 29.7±1.7 | 0 |
| 10-12 | 30 | | | 10.3±1.4 | — | — | — | — | |
| 12-14 | 72 | | | 10.2±1.2 | — | — | — | — | 0 |
| cont. | 18 | | | 11.0±0.8 | 6 | | | 28.2±2.2 | 0 |

and 7-8 group although the ratio was low.

In Experiment III, the masculinization ratio was high in 3 groups 0-8, 1-9 and 2-10. In the 3-11 group for which the treatment began on the 3rd week, the ratio decreased by 1/3 compared with that for the previous 3 groups. Males did not appear in the test group for which the treatment started after the 4th week.

For all the 7 test groups in Experiment IV, the masculinization ratios were 0 %. Masculinization was not observed in the 2 week-treatment with 0.8 ppm. p. 11

Masculinization ratios obtained by 4 experiments are given in Fig. 5. With regard to the duration of MT treatment, from the results of Experiment I, a high ratio of reversal was observed as the duration became longer over a range of up to 8 weeks (Fig. 5a). During a short treatment period of 1 or 2 weeks, a slight reversal was observed with 5 ppm (Fig. 5b).

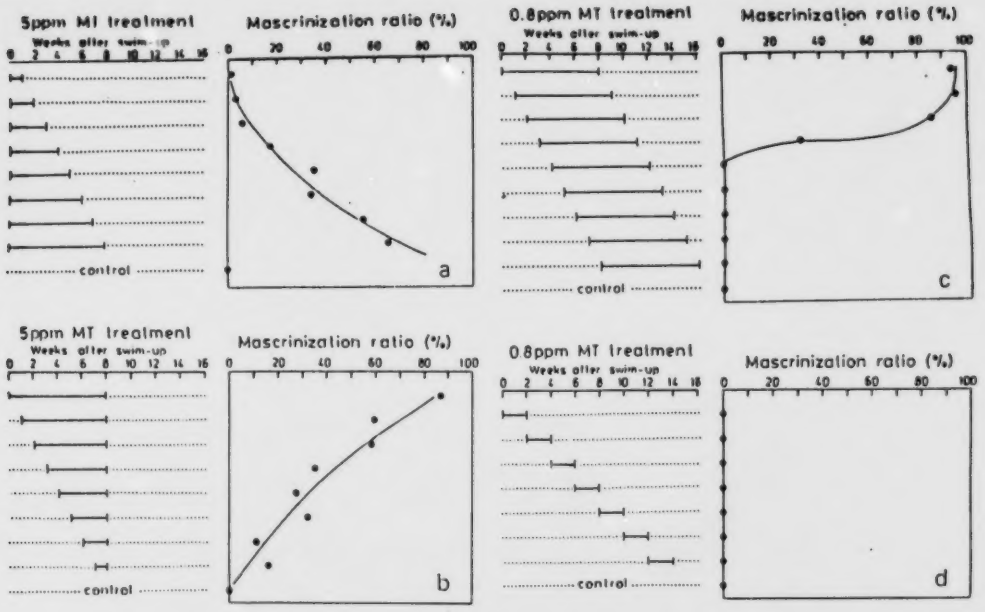


**Fig. 5.** Musculinization ratio (MR) of 5 ppm and 0.8 ppm methyltestosterone (MT) treatment for various periods. In calculation the MR, male was evaluated as 1.0 and hermaphrodite was evaluated as 0.5. (a) Test group treated with MT during a week to 8 weeks from the swim-up stage. (b) Test groups treated with 5 ppm MT from various time to 8th week after the swim-up. (c) Test groups treated with 0.8 ppm MT during various 8 weeks.
(d) Test groups treated with 0.8 ppm MT during various 2 weeks.
Solid line and broken line represent duration of MT treatment and normal feeding, respectively.

With 0.8 ppm, no effect was observed in the 2 week-treatment (Fig. 5d). It became clear that it was necessary to have a long period of treatment. A high ratio of reversal was observed when the 8 week-treatment was continued even when the start of treatment was delayed until after the 2nd week from swim-up. It also became clear that when the start of treatment was delayed for 4 weeks with 0.8 ppm, the reversal did not occur even when the MT treatment was given after that time (Fig. 5c). With 5 ppm treatment, a low rate of

masculinization was observed in a group for which the treatment was given for 1 week from the 7th week. Therefore it is thought that differences with regard to the treatment time may occur according to differences in the MT concentration.

3. Dosage level of androgen and the masculinisation ratio

In order to study effects of androgen on sex differentiation, the fluctuations in the sex distribution was observed during the oral administration of androgen at various dosage levels from the swim-up stage.

1) Sample fish and methods

All-female populations obtained by mating sex-reversed males (XX males) with normal females were used for sample fish. 17$\alpha$-methyltestosterone(MT) and 11ketotestosterone (11 KT, Sigma CO.) were used for androgen. They were dissolved in ethanol and added to the assorted feed sold on the market. Sample fish were fed by oral administration for 8 weeks from swim-up.

Two experiments were carried out for the MT treatment test. In Experiment I, 0.01 ppm and 0.05 ppm were set as standard concentrations. Groups of concentration of 10, $10^2$, $10^3$ and $10^4$ times of each standard concentration were set up and the effective range of concentration for the induction of sex differentiation was studied. In Experiment II, more divisions of groups were set up for the effective range of concentration obtained by Experiment I. 12 groups of

concentration in Experiment I are 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 500 ppm, an ethanol control group and a control group without treatment. 10 groups of concentration in Experiment II are 0.1, 0.3, 0.4, 0.5, 0.6, 0.8, 1, 500, 1,000 ppm and a control group. After an 8 week-period of p. 12 treatment, ordinary assorted feed was given. In experiment I, breeding was continued up to the 39th month and in Experiment II, up to the 49th month and the masculinization ratio was studied. The number of sample fish was 205 for Experiments I and II respectively.

11KT was tested with 10 groups of concentration of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100 ppm and a control group. After treatment, normal breeding was continued up to the 26th month and the masculinization ratio was studied. The number of sample fish was 105.

A portion of sample fish was collected from each group of MT and 11KT at one year after swim-up and at the end of the test. Then the growth and the sex ratio were studied and the masculinization ratio was obtained. The breeding conditions during the treatment were the same as those for the test conducted for the treatment time described in the previous section.

2) Results

(1) Dosage level of the MT treatment and the masculinization ratio

The sex ratio and the masculinization ratio obtained

**Table 6.** Sex distribution and masculinization ratio (M. R.) at various methyltestosterone dosage levels examined 1, 3 and 4 years after swim -up. F ; female, H ; hermaphrodite, M ; male

| Treatment | Sex distribution | | | | | | | | | MR |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 year | | | 3 year | | | 4 year | | | |
| | F | H | M | F | H | M | F | H | M | |
| ppm | | | | | | | | | | % |
| 1000 | 51 | 3 | 11 | 4 | | | | | | 15.9 |
| 500 | 38 | 1 | 1 | | | | 4 | | | 3.4 |
| 500 | 89 | 1 | 11 | 23 | | 1 | | | | 10.0 |
| 100 | 42 | | 3 | | | | 4 | 2 | | 7.8 |
| 50 | 48 | 2 | | | | | 6 | | | 1.8 |
| 10 | 32 | 6 | 12 | | | | 2 | 7 | | 31.4 |
| 5 | 21 | 8 | 21 | | | | | 7 | 6 | 54.8 |
| 1 | 9 | 5 | 32 | | | | 6 | 2 | 8 | 70.2 |
| 1 | 21 | 1 | 87 | 6 | | 17 | | | | 92.5 |
| 0.8 | 2 | 2 | 85 | 7 | 2 | 17 | | | | 90.8 |
| 0.6 | 2 | | 49 | 2 | | 13 | | | | 93.4 |
| 0.5 | 3 | 4 | 28 | | | | | 4 | 20 | 88.1 |
| 0.5 | 1 | 1 | 45 | 1 | 2 | 28 | | | | 91.4 |
| 0.4 | 6 | 1 | 58 | 10 | | 7 | | | | 79.9 |
| 0.3 | 13 | 2 | 65 | 2 | 2 | 8 | | | | 81.9 |
| 0.1 | 50 | | | | | | 10 | | 5 | 7.7 |
| 0.1 | 40 | 4 | 18 | 15 | 1 | 6 | | | | 31.5 |
| 0.05 | 45 | | | | | | 6 | | | 0.0 |
| 0.01 | 43 | | | | | | | | | 0.0 |
| alcholcont. | 53 | | | | | | | | | 0.0 |
| cont. | 18 | | | 6 | | | | | | 0.0 |
| cont. | 60 | | | | | | | | | 0.0 |

from Experiments I and II are given in Table 6. Body lengths of 1, 3 and 4 year-old fish are given in Table 7. The lowest concentration at which masculinization was observed was 0.1 ppm. No male appeared at below 0.05 ppm. At above 0.3 ppm, it was observed that the masculinization ratio increased with increase in the concentration. The highest ratio for Experiment I was 88.1 % at 0.5 ppm and that for Experiment II was 93.4 % at 0.6 ppm. At higher concentrations than those stated above, the masculinization ratio decreased with

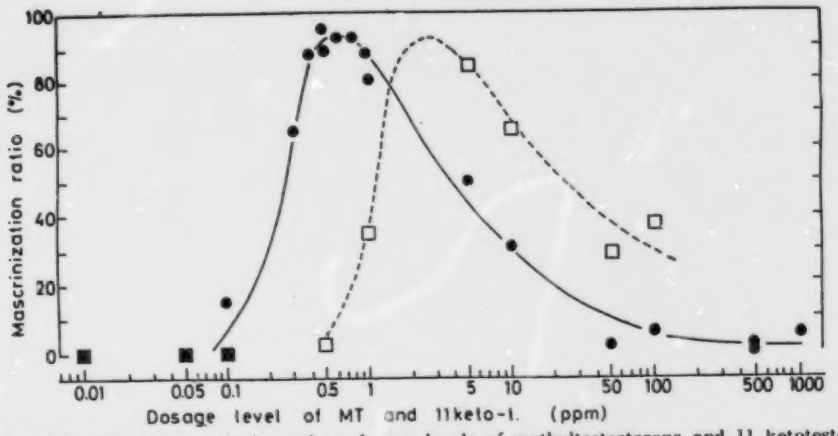


**Fig.6.** Musculinization ratio in various dosage levels of methyltestosterone and 11 ketotestosterone. Solid circle and solid line represent MT dosage. Square and broken line represent 11 keto-t. dosage

increase in the concentration and it became very low at over 50 ppm. The relationship between the dosage level and the masculinization ratio showed a curve with a sharp peak at 0.5-1 ppm. The range of concentration for 50 % masculiniaation ratio was 0.3-5 ppm (Fig. 6). p. 13

No difference in the growth of body length was observed at different concentrations in Experiments I and II. It is thought that there is no effect on growth by the treatment at 0.01-1,000 ppm (Table 7).

(2) Dosage level of the 11ketotestosterone treatment and the masculinization ratio

The ratio, the body length and the masculinization ratio on the 12th month and the 26th month after swim-up are given in Table 8. The lowest concentration at which masculinization was observed was 0.5 ppm. The highest ratio was

**Table 7.** Body length at various mthyltestosterone dosage levels at various months after swim -up

| Treatment | Body length±SD | | | |
|---|---|---|---|---|
| | 10 mo. | 11 mo. | 39 mo. | 49 mo. |
| ppm | cm | cm | cm | cm |
| 1000 | | 12.4±1.3 | | |
| 500 | 10.2±1.8 | | 23.1±1.8 | 28.3±1.9 |
| 500 | | 11.7±1.0 | 21.9±1.3 | |
| 100 | 10.3±4.0 | | | 30.0±2.3 |
| 50 | 9.1±1.7 | | | 30.0±2.8 |
| 10 | 9.5+1.1 | | | 30.2±1.3 |
| 5 | 11.4±4.8 | | | 29.5±2.3 |
| 1 | 10.5±1.4 | | | 27.6±3.9 |
| 1 | | 11.3±0.9 | 22.9±1.6 | |
| 0.8 | | 10.6±1.2 | 22.7±1.7 | |
| 0.6 | | 10.2±1.2 | 22.0±1.9 | |
| 0.5 | 9.9±1.3 | | | 29.0±1.8 |
| 0.5 | | 11.2±1.8 | 23.1±1.5 | |
| 0.4 | | 10.1±1.1 | 23.0±1.0 | |
| 0.3 | | 10.7±0.9 | 23.2±1.3 | |
| 0.1 | 10.6±1.4 | | | 27.4±4.3 |
| 0.1 | | 11.1±1.3 | 23.3±2.2 | |
| 0.05 | 10.0±1.8 | | | |
| 0.01 | 9.7±1.7 | | | |
| alcholcont. | 9.7±1.4 | | | |
| cont. | 9.3±1.8 | | | |
| cont. | | 11.0±0.8 | 22.8±1.6 | |

83.8 % at 5 ppm. Decrease in the masculinization ratio in the group of high concentration of 11KT was observed as seen in the case of MT. However, in the range up to 100 ppm, the decrease was not that conspicuous. The relationship between the concentrations of the treatment and the masculinization ratio is shown in Fig. 6 by a curve with a single peak similar to that for MT. The concentration range which showed masculinization of over 50 % was thought to be that of 2 ppm-10 ppm.

**Table 8.** Musculinization ratio (M. R.) at various 11 ketotestosterone dosage levels

| Treatment | 12 mo. after swim-up | | | | 26 mo. after swim-up | | | | M. R. |
|---|---|---|---|---|---|---|---|---|---|
| | F | H | M | BL±SD | F | H | M | BL±SD | |
| ppm | | | | cm | | | | cm | % |
| 100 | 8 | 2 | 5 | 13.6±2.4 | 5 | | 2 | 18.6±1.7 | 36.4 |
| 50 | 11 | 2 | 6 | 13.3±1.8 | 10 | 5 | | 19.1±2.3 | 27.9 |
| 10 | 4 | | 16 | 13.6±1.5 | 8 | | 6 | 19.3±2.8 | 64.7 |
| 5 | 1 | 1 | 19 | 13.2±1.7 | 4 | | 9 | 17.7±1.6 | 83.8 |
| 1 | 10 | | 6 | 11.6±1.6 | 11 | | 4 | 17.8±2.1 | 34.3 |
| 0.5 | 19 | 4 | | 11.8±2.3 | 16 | | | 17.0±2.0 | 2.3 |
| 0.1 | 20 | | | 11.8±1.8 | 10 | | | 16.1±1.6 | 0.0 |
| 0.05 | 21 | | | 12.4±1.4 | 15 | | | 16.0±1.6 | 0.0 |
| 0.01 | 15 | | | 13.0±1.7 | 8 | | | 21.5±1.9 | 0.0 |
| cont. | 15 | | | 14.3±2.3 | 20 | | | 22.1±2.8 | 0.0 |

The best growth of body length was observed in the control group. However, the difference between that and the treated groups was small. No correlation between the dosage level and the growth was observed (Table 8).

4. Maturation characteristics of sex-reversed males and the sex ratio in the $F_1$ generation p. 15

In order to study the functional maturation of the individuals whose sex differentiation was reversed from genetic females to males by androgen, the developmental stages of the gonad were examined at spawning time, the 32nd month after hatching. The milt from an individual mature male was mated with a normal female and the sex ratio in the $F_1$ generation was observed. The type of sex chromosomes of rainbow trout was studied.

1) Sample fish and methods

24 sample fish were studied from the group which was treated by 1 ppm of MT and in which a high ratio of

masculinization was observed in the experiment of the MT dosage level. The stages of maturation were studied in the spawning period, the 32nd month after hatching. Collectability of the milt by squeezing out, the stage of formation of the spermiduct, the shape of gonad and the gonad somatic index were studied and the maturation function of the sex-reversed males was observed.

In order to study the fertility and type of sex chromosomes of the individual masculinized fish produced by MT treatment, the milt from each individual male was mated with a normal female and the sex ratio in the $F_1$ generation was observed.

A mixture of genetic males and females was used for sample fish. MT was added to the feed and the sample fish were fed by oral administration for 60 days from swim-up. After the treatment, they were given normal feed and bred up to the 36th month after hatch-out. Concentrations of MT treatment were 1, 5, 10, 50 and 100 ppm. A control group was added to these groups. Other breeding conditions were the same as those used for the test of administration time.

On the 36th month, males which showed secondary male sexual characteristics in the maturation period and from which the milt could be squeezed out were chosen. 65 fish from the groups treated by MT (23 from the 1 ppm group, 5 from the 5 ppm group, 17 from the 10 ppm group, 18 from the 50 ppm group, 2 from the 100 ppm group) and 5 from the control

group, a total of 70 fish was mated with normal females individually. The sex ratio of each $F_1$ generation was studied on the 10-13th month after hatching. Of the 70 pairs mated, the fertility ratio was studied at the 16th hour after fertilization for 5 pairs from the 1 ppm group, 5 pairs from the 10 ppm and 5 pairs from the control group.

For 2 hermaphrodites from the 1 ppm group and 3 from the 10 ppm which appeared, the gonads were extracted and mated with normal eggs using the testes part. Then the fertilization ratio and the sex ratio in the $F_1$ generation were observed.

2) Results

i) Maturation characteristics of sex-reversed fish

The maturation stages of 24 fish in the sample group treated by 1 ppm of MT (treated for 8 weeks from swim-up) are given in Table 9. Most of them showed secondary male sexual characteristics. Immature fish were small in number, 4 males and 2 females. 17 mature males appeared. They were divided into 3 types: (1) 8 males with normal form of testes such that the milt could be collected by squeezing out (mature male type 1, Plate I, 5), (2) 4 males with compressed testes with immature or blocked genital ducts from which the milt could not be squeezed out (mature male type 2, Plate I, 6) and (3) 5 males with unbalanced testes from which the milt could not be squeezed out (mature male type 3, Plate II, 7). Normal males whose milt could be squeezed out constituted

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**Table 9.** Sexual character of the fish treated by 1 ppm methyltestosterone at 32 months old

| Gonadal sex | Secondary sexual cfaractor | Milt squeezed out | N | BW±SD | BL±SD | GSI±SD |
|---|---|---|---|---|---|---|
| | | | | g | cm | % |
| Mature male[1] | + | + | 8 | 464.1±72.7 | 29.3±0.6 | 2.6±0.6 |
| Mature male[2] | + | − | 4 | 463.0±42.4 | 29.8±1.7 | 2.5±1.0 |
| Mature male[3] | + | − | 5 | 479.0±53.7 | 30.0±1.6 | 1.7±0.8 |
| Immature male | − | − | 4 | 444.2±48.0 | 29.8±1.0 | 0.3±0.1 |
| Immature female | − | | 2 | 414.4±89.2 | 29.8±3.2 | 0.2±0.04 |
| Hermaphrodite | + | − | 1 | 392 | 29.9 | 1.5 |

1. male with normal mature testes (milt could be squeezed out.)
2. male with compressed testes lacking for genital duct.
3. male with unbalanced testes.
GSI. gonad somatic index (gonad weight/body weigt × 100)

50 % of mature males. Mature male type 2 and type 3 formed sperm. However, the seminal fluid did not reach the germinal orifice due to lack of a spermiduct or blockage in the rear. One hermaphrodite was observed. The sperm formation in the gonad area was observed and the secondary male sexual characteristics appeared. However, the spermiduct was immature and the milt could not be squeezed out. The ovary area was immature (Plate II, 8).

GSI for mature male types 1 and 2 were 2.5-2.6 % and normal. For male type 3, it was 1.7 % and somewhat low.

The weight and body length measured on the 32nd month were of standard values. No morphological difference was observed in the gonads.

ii) Sex ratio in the $F_1$ generation

The sex ratio of the groups treated by MT on the 36th month after hatching and that of the $F_1$ generation are given in Table 10.

**Table 10.** Sex distribution of methyltestosterone-treatment 36 months after hatch and sex ratio of $F_1$ generation produced by mating mature male with normal female
F; mature female, H; hermaphrodite, M; mature male, Im; immature (sex unknown)

| MT treatment | Sex distribution at 36 mo. | | | | Percentage of male | No. of progeny test | Sex ratio of $F_1$ | |
|---|---|---|---|---|---|---|---|---|
| | F | H | M | Im | | | All female | 1 |
| ppm | | | | | % | | | |
| 1 | 22 | 2 | 70 | 11 | 74.4 | 23 | 2 | 21 |
| 5 | 5 | | 9 | 4 | 64.3 | 5 | 0 | 5 |
| 10 | 23 | 3 | 49 | 8 | 65.3 | 17 | 2 | 15 |
| 50 | 35 | 1 | 42 | 3 | 53.8 | 18 | 1 | 17 |
| 100 | 18 | 1 | 19 | 6 | 50.0 | 2 | 0 | 2 |
| cont. | 37 | | 41 | 6 | 52.6 | 5 | 0 | 5 |

**Table 11.** Sex distribution of $F_1$ generation producted by mating sex-reversed male with normal female.
F; female M; male

| MT treatment of father | Mating No. | Sex distribution of $F_1$ | | Significance of difference of ratio from 1 : 1 |
|---|---|---|---|---|
| | | F | M | |
| ppm | | | | |
| 1 | 1 | 31 | 0 | ** |
| | 2 | 9 | 0 | ** |
| 10 | 1 | 51 | 0 | ** |
| | 2 | 45 | 0 | ** |
| 50 | 1 | 47 | 0 | ** |

** : $p<0.005$.

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The male ratio in mature fish was highest at 74.4 % at 1 ppm followed by 53.8 % and 50.0 % at 50 ppm and 10 ppm respectively and was the same as that for the control group. The sex ratio of the $F_1$ generation produced by mating a sex-reversed male with a normal female in the groups treated by MT, 2 males from 23 in the 1 ppm group, 2 from 17 in the 10 ppm group, 1 from 18 in the 50 ppm group, a total of 5 males produced all-female $F_1$ (Tables 10 and 11). 5 males from

**Table 12.** Sex ratio of $F_1$ generation producted by mating hermaphrodite appeared in methyltestosterone 1 ppm and 10 ppm treated groups, with normal females
F; female M; male

| MT treatment of father | Mating No. | Sex distribution of $F_1$ | | Significance of difference of ratio from 1 : 1 |
|---|---|---|---|---|
| | | F | M | |
| ppm | | | | |
| 1 | 1 | 24 | 0 | ** |
| | 2 | 138 | 0 | ** |
| 10 | 1 | 17 | 0 | ** |
| | 2 | 50 | 0 | ** |
| | 3 | 50 | 0 | ** |

** : $p<0.005$

the 5 ppm group and 2 from the 100 ppm produced a 1:1 sex ratio in $F_1$ and 5 from the control group produced a 1:1 ratio in $F_1$. In the treated groups, 5 males out of 65 produced all-female $F_1$ and 60 produced a 1:1 sex ratio of $F_1$. 5 males which sired all female progenies were considered to be the males induced from the genetic females by the MT treatment.

The sex-reversed males(XX) showed secondary male sexual characteristics and could not be distinguished from the XY males by appearance (Plate II, 9).

Table 12 shows the sex ratio in $F_1$ produced by fertilization by sperms obtained from each of 5 hermaphrodites which appeared in the MT 1 ppm group and 10 ppm group. $F_1$ from 5 pairs consisted of all females. It became clear that these hermaphrodites were produced by incomplete induction to males from genetic females.

These results demonstrated that the sex chromosomes p. 17
of rainbow trout were of female homogamety and that they agreed with the sex ratio of $F_1$ produced by functional sex-reversed males from genetic females. The sex chromosomes of 5 males which produced all females and those of 5 hermaphrodites were XX. It was confirmed genetically that they were reversed to individuals having male functions by oral administration of MT. Okada et al.(1979) and Johnstone et al.(1979) have already reported that the sex chromosomes of rainbow trout were of female homogamety and the same result was obtained in our present study.

5. Maturation of all-female population in the $F_1$ generation and the sex ratio of the $F_2$ generation

In order to study methods of producing all females using XX males which were reversed to males from genetic females by the MT treatment, the stages of maturation of the all-female population and the sex ratio of $F_2$ were observed.

1) Sample fish and methods

From the all-female $F_1$ populations which appeared in the MT 1 ppm group and the 10 ppm group, one from each group was bred normally until maturation. The stages of maturation were studied and they were mated with normal males. Then the sex ratio of $F_2$ was studied.

In order to study the stages of maturation, the egg weight and GSI were measured on the 46th month after hatching

**Table 13.** Reproductive characteristics of all females ($F_1$) produced by mating sex-reversed male (XX) with normal female at the age of 46 months

| Mature female | N | Egg weight ± SD | GSI ± SD |
|---|---|---|---|
| | | mg | % |
| All females sired by XX males | 12 | 52.91 ± 6.69 | 15.53 ± 2.01 |
| Normal ferales | 10 | 50.10 ± 5.84 | 15.77 ± 1.28 |

GSI : gonad somatic index (gonad weight/doay weight)

which is the maturation period for an all-female population made of 12 females which were produced by males treated by 1 ppm of MT. For comparison, the same measurements were taken for 10 normal females of the same first year which were bred in the same breeding pond. Ratios of fertilized and eyed eggs were studied for 5 females in the all-female population.

For 12 all females from the MT 1 ppm $F_1$ and 15 all females from the MT 10 ppm $F_1$, a total of 27 mature females, eggs from each fish were fertilized with mixed sperm collected from normal males and the sex ratio of the $F_2$ generation was studied between the 6th-8th month after hatching.

2) Results

i) Maturation characteristics of the all-female population in $F_1$

The egg weight(mg/egg) at the time of ovulation and GSI were measured for the all-female population of 12 in the MT 1 ppm $F_1$ and for 10 normal females. Results are given in Table 13. The weight was 52.9 mg for the all-female population and 50.1 mg for the control group. No significant

**Table 14.** Rates of fertilized and eyed eggs in mating females sired by XX male with normal males

| Mating No. | Ratio of fertilized egg | Ratio of eyed egg |
|---|---|---|
| | % | % |
| 1 | 95 | 82 |
| 2 | 100 | 91 |
| 3 | 100 | 78 |
| 4 | 90 | 85 |
| 5 | 100 | 73 |

**Table 15.** Sex ratio of $F_2$ generation produced by mating females ($F_1$) sired by XX males, with normal males

| MT treatment of P generation | No. of progeny test | Sex ratio | |
|---|---|---|---|
| | | 1 | other ratio |
| ppm | | | |
| 1 | 12 | 12 | 0 |
| 10 | 15 | 15 | 0 |

difference was observed. GSI was 15.5 % for the former and 15.8 % for the latter. No difference in the characteristics was observed between females sired by XX males and normal females.

Ratios of fertilized and eyed-eggs are given in Table 14. The ratio of fertilized eggs for 5 females was a high ratio of more than 90 % and that of eyed-eggs 73-92 %, was also high.

ii) Sex ratio of the $F_2$ generation of all-female population in $F_1$

The sex ratio of the $F_2$ generation of the all-female p. 18
population of 12 in the MT 1 ppm $F_1$ and that of 15 in the 10

ppm $F_1$ are given in Table 15. The sex ratio of the $F_2$ generation of 27 females was 1:1. It was confirmed that these sex chromosomes were of XX type. It was also confirmed that the all-female population sired by XX males had the same reproductive functions and the same fertilization and hatching results as normal females. It became clear that there was no problem in utilizing these females as breeding stocks.

6. Discussion

Takashima et al.(1980) studied the sex differentiation of rainbow trout and stated that the reproductive cells reached the gonadal ridge at hatch-out and the meiosis could be observed at swim-up. The same results were obtained in our experiments. It was suggested that the sex differentiation of rainbow trout begins from swim-up. Nakamura et al. (1974) stated that the sex differentiation of masu salmon starts about 5 weeks from hatch-out. However, the swim-up time is on the 4th week, therefore sex differentiation can begin in the same period. It is thought that there is not a great difference between the two species.

In the administration of sex steroids for the artifical masculinization of rainbow trout, a reversal of sex differentiation by an 8 week-treatment from swim-up has been reported (Okada et al., 1979, Johnstone, 1978, 1979). From test results of various times and periods of administration, it became clear that the treatment is effective when the

administration of MT is started from swim-up or within 2 weeks from swim-up and continued for 8 weeks. Yamamoto (1969) stated that the administration of sex steroids for the reversal of sex differentiation should begin in the period before the sex differentiation and should be continued until the sex differentiation is completed. The sex differentiation of rainbow trout begins at the time of swim-up, however, there are only a few cells of the meiotic period until the 2nd week from swim-up. It was made clear in this study that the meiotic cells increased after the 4th week from swim-up. From the study of the experimental results of the period of administration of MT and the tissue change of the oogonia, the number of meiotic cells at the time of swim-up was small. Judging from the fact that until the 2nd week mainly an increase in the oogonia is observed, it is assumed that progress in the sex differentiation is slow during the 2 weeks from swim-up. The oocytes of the meiotic period increased on the 4th week. Therefore it is thought that the administration of MT after this time is not effective. Thus it is thought that the transitional period from the oogonia to the oocytes has a significant meaning in sex differentiation.

From the tests for the concentrations in 2 kinds of androgen administered and the masculinization ratio, it became celar that the effective concentrations for treatment were limited to a narrow range and that the masculiniaation ratio decreased rapidly outside the range of either high or

low concentrations. The most effective dosage level of MT was 0.5-1 ppm which yielded more than 90 % of masculinization. This level of concentration is the same as that of 1 ppm for rainbow trout reported by Yamazaki(1976) and Okada et al. (1979) and is also close to that of 3 ppm reported by Johnstone et al.(1979). The effective level of concentration for medaka (Yamamoto, 1958), gold fish (Yamamoto and Kajishima, 1968) and Tilapia (Clemens and Inslee, 1968) is thought to be 25-30 ppm but that for rainbow trout is low. The reason is not known yet but it is thought that there is a relationship with the difference in the MT intake due to difference in the amount of feed besides the difference between cold-water species and warm-water species.

The most suitable concentration of 11KT was thought to be about 5 ppm which was about 10 times that of MT. Masculinization of close to 100 % was obtained by the administration of 25 ppm of MT for medaka (Yamamoto, 1958) and that of 200 ppm of 11KT (Hishida and Kawamoto, 1970). The dosage p. 19 level of 11KT was about 8 times that of MT.

The same results were obtained for rainbow trout. Thus it was assumed that these 2 kinds of sex steroids had a similar physiological action for both species.

From the tests concerning the periods and concentrations of administration, it was confirmed that the most effective treatment method for masculinization of rainbow trout was to begin the administration of 0.5-1 ppm of MT at

the time of swim-up or within 1-2 weeks from swim-up and continue it for 8 weeks. No deaths or effects on growth were observed and it is thought to be a safe and reliable method.

It is thought that there is no abnormality in the sperm formation of sex-reversed males. The hatching result was normal. No abnormalities such as an appearance of abnormal fish were observed. Therefore it is thought that there is no difficulty in the production of X sperms from the XX reproductive cells. However, a high ratio of morphological abnormalities in the testes and spermiducts appeared. Takahashi(1978) stated that the excessive administration of sex steroids causes problems in the morphological formation of entire reproductive organs. The results obtained in the present study are thought to be caused by inadequate MT administration. It is necessary to study further the duration and concentrations of administration in order to answer these questions. Furthermore it is thought that the somatic element of the gonad and the sex differentiation of the reproductive cells should be studied separately and that the effects of sex steroids on these should also be studied.

It was confirmed that all females in $F_1$ sired by XX males were completely normal genetically and in reproductive function. It became clear that there is no problem in utilizing them for all-female stocks.

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## Chapter III Feminization of genetic males

Estrogen treatment was tested in order to confirm YY males in $F_1$ produced by sex-reversed females which were feminized from genetic males. YY males are thought to produce all males when mated with normal females. Production methods of all males by utilizing YY males were also discussed.

### 1. Process of sex differentiation of genetic males

Process of sex differentiation of males was observed histologically in order to study feminization of genetic males.

#### 1) Sample fish and methods

Genetic all males obtained by mating YY males with normal females were used for samples. Specimens were collected regularly from the time of hatch-out to the 25th week after swim-up. Specimens were fixed in Bouin's solution and paraffin sections were made. The sections were stained with Delafield hematoxylin-eosin and examined under a microscope. The breeding water temperature was 10.5°C.

#### 2) Observation results

Development of the gonad at various stages is described below.

Hatch-out (total effective temperature 320°C): The gonad is observed directly below the kidney and its diameter was about 20 $\mu$m. 1-2 large and round spermatogonia of cell diameter 15-17 $\mu$m were observed surrounded by a layer of somatic cells. Morphology at this time was the same as that

of females and no difference was observed.

Swim-up (total effective temperature 590-610°C): The gonad developed to about 30 x 50 μm. Many somatic cells were observed in the gonad. 1-2 reproductive cells existed and showed the same morphology as that at the time of hatch-out (Plate II, 10).

6 weeks after swim-up (total effective temperature 1,041°C): The gonad became a little larger (50-70 μm) compared with that at the time of initial swim-up. The spermatogonia decreased to cell diameter of 10-15 μm and their number increased. Development of tubular tissue was observed at the base.

8 weeks after swim-up (total effective temperature 1,190°C): The size of the gonad did not change greatly (50 x 80 μm). The number of reproductive cells somewhat increased but no great change was observed from those of 5 weeks (Plate II, 11). p. 20

25 weeks after swim-up (total effective temperature 2,440°C): The gonad grew to a large size, 300-500 μm. Reproductive cells were the spermatogonia alone, however, the development of the seminiferous tubule and the cyst formation of the spermatogonia were observed. A testes-like form became apparent (Plate II, 12).

From these observation results, no difference from male to female was observed in the gonad at the time of hatch-out and the stage was that of no differentiation. In the

gonad at the time of swim-up, the increase of somatic cells was somewhat greater compared with that of females. The change in the reproductive cells which was observed in females was not observed even at the 25th week after swim-up. It was demonstrated that the testes-like formation was characterized by a development of somatic elements such as the seminiferous tubule and the spermiduct.

2. Dosage level of estrogen and the feminization ratio

In order to study effects of oral administration of estrogen on the sex differentiation, estrogen of various concentrations was administered orally from the time of swim-up and the fluctuations in the sex ratio were observed.

1) Sample fish and methods

Genetic all males obtained by mating YY males with normal females were used for samples. Estradiol-17$\beta$ ($E_2$) was used for estrogen. It was added to the feed sold on the market after being dissolved in ethanol and given by oral administration for 8 weeks from swim-up. Concentrations of $E_2$ in the feed were 10, 50 and 100 ppm. Tests were carried out using 3 groups of different concentrations and 1 control group, a total of 4 groups. The experimental conditions such as the adding method of $E_2$ to the feed sold on the market, the feeding method and the breeding water temperature were the same as those for the tests for the duration of administration of MT. The number of sample fish was 105 for

each group.

After the treatment, they were given normal feed and bred up to the 15th month after swim-up. Then the sex ratio was studied. Sex was determined by crushing raw specimens and by examining them under a microscope. A fish with an ovary was determined as female, that with testes as male and that with both tissues as hermaphrodite. When the determination was difficult, a tissue section was made and then identified.

2) Results

During the period of $E_2$ administration from swim-up until the 8th week, no difference due to dosage level was observed in mortality and body growth. Each test group showed a high rate of survival and the same growth result as that of the control group.

The number of appearance of males and females, body length and feminization ratio 15 months after swim-up are given in Table 16. The induction effect of estrogen on sex differentiation was expressed as the feminization ratio. The feminization ratio was calculated by multiplying the number of appearance of females by 1 and that of hermaphrodites by 0.5 and then dividing by the total number of fish. The results are expressed as a percentage.

The highest feminization ratio was 14.1 % for the 50 ppm group but all other groups had a low ratio except one female appeared from 45 fish in the 100 ppm group. In the

**Table 16.** Sex distribution and feminization ratio (F. R.) at various dosage levels of estradiol-17 $\beta$ examined 15 months after swim-up. F; female, H; hermaphrodite, M; male

| Treatment | F | H | M | FR | BL±SD |
|---|---|---|---|---|---|
| ppm | | | | % | cm |
| 100 | 1 | | 44 | 2.3 | 12.0±1.9 |
| 50 | 4 | 3 | 32 | 14.1 | 11.8±3.1 |
| 10 | | | 19 | 0.0 | 13.0±1.7 |
| cont. | | | 26 | 0.0 | 11.6±1.3 |

50 ppm group, 3 hermaphrodites (7.7 %) appeared for 4 females (10.3 %). The method of $E_2$ administration used in this test yielded a low feminization result.

No difference was observed in the body lengths on the 15th month between the $E_2$ treated groups and the control group. Considering the survival ratio and growth results during the period of treatment, it is thought that there is no change in the breeding results produced by the administration of $E_2$.

### 3. Sex ratio of the $F_1$ generation of sex-reversed females

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In order to study individuals which were reversed to functional females from genetic males by estrogen and the sex chromosomes of rainbow trout, we observed the sex ratio of the $F_1$ generation produced by mating the eggs from mature females appeared in the estrogen treated groups with normal males individually.

1) Sample fish and methods

Ordinary rainbow trout of female and male constituting a mixed group were used for samples. Estrone (EST, Iwaki Seiyaku) was used for estrogen. Addition of EST to the feed was done using ethanol in the same manner as in the other administration tests. The oral administration was carried out for 8 weeks from the time of swim-up. The EST concentrations were 1, 10, 50, 100 and 200 ppm. The test groups were 5 groups at these concentrations and a control group, a total of 6 groups. The number of sample fish was 205 for each group. Conditions for breeding experiment were the same as those for the other experiments. After the treatment period was over, they were given ordinary mixed feed sold on the market and bred up to the 36th month after hatching. Then males and females were identified for mature fish. The feminization effects of EST were studied by observing the female ratio in mature fish. The sex ratio of $F_1$ was observed in order to confirm sex-reversed fish and to study the sex chromosomes of rainbow trout. 2 mature females from the 10 ppm group, 4 from the 50 ppm group, 9 from the 100 ppm group, 2 from the 200 ppm group and 3 from the control group were chosen for samples. The eggs from each mature female were mated with normal males and the sex was determined on the 10-13th month from hatching.

2) Results

i) Fluctuations in the female ratio by EST administration

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Table 17. Sex distribution in estrone-treated groups and No. of progeny test at 36 months after hatch out. F; female, H; hermaphrodite, M; male, Im; immature

| EST treatment | Sex distribution at 36 mo. F | H | M | Im | Percentage of female | No. of progeny test |
|---|---|---|---|---|---|---|
| ppm | | | | | % | |
| 1 | 34 | | 34 | 18 | 50.0 | |
| 10 | 28 | | 32 | 10 | 46.7 | 2 |
| 50 | 23 | 1 | 18 | 6 | 54.8 | 4 |
| 100 | 47 | | 38 | 3 | 55.3 | 9 |
| 200 | 30 | | 21 | 6 | 58.0 | 2 |
| cont. | 37 | | 41 | 6 | 47.4 | 3 |

The number of mature males and females which appeared on the 36th month is given in Table 17. The ratio of mature fish including both males and females was 79.5-96.6 %, mean 89.6 % respectively. About 10 % of the fish observed were immature. The female ratios for mature fish were 50.0 % and 46.7 % in the 1 ppm group and the 10 ppm group respectively and no increase in the female ratio was observed. Those in the 50 ppm group and the 100 ppm group were about 55 % and in the 200 ppm group, 58 %. Increase in the female ratio in the groups at high concentrations was observed, however, the range of increase was small. The number of hermaphrodites was also small and only one appeared in the 50 ppm group (Plate III, 13).

The number of appearance of males and females in this experiment had no significant difference from the sex ratio of 1:1. It was thought that the feminization effects by EST was low.

**Table 18.** Sex distribution of $F_1$ generation produced by mating estrone -treated females with normal males at 10-13 months after hatch out

F; female, M; male

| EST treatment of matuer fish | Mating No. | Sex distributitn of $F_1$ | | Significance ofdifference of matio (F : M) from: | | |
|---|---|---|---|---|---|---|
| | | F | M | 1: | 1:2 | 1:3 |
| 10 ppm | 1 | 32 | 30 | NS | * | ** |
| | 2 | 21 | 20 | NS | ** | ** |
| 50 ppm | 1 | 6 | 6 | NS | ** | ** |
| | 2 | 56 | 59 | NS | ** | ** |
| | 3 | 12 | 11 | NS | ** | ** |
| | 4 | 36 | 32 | NS | ** | ** |
| 100 ppm | 1 | 42 | 39 | NS | ** | ** |
| | 2 | 14 | 13 | NS | ** | ** |
| | 3 | 14 | 9 | NS | ** | ** |
| | 4 | 51 | 51 | NS | ** | ** |
| | 5 | 10 | 11 | NS | ** | ** |
| | 6 | 9 | 13 | NS | ** | ** |
| | 7 | 9 | 9 | NS | ** | ** |
| | 8 | 38 | 78 | ** | NS | NS |
| | 9 | 18 | 51 | ** | NS | NS |
| 200 ppm | 1 | 60 | 64 | NS | ** | ** |
| | 2 | 25 | 76 | ** | NS | NS |
| cont. | 1 | 32 | 29 | NS | ** | ** |
| | 2 | 75 | 77 | NS | ** | ** |
| | 3 | 23 | 23 | NS | ** | ** |

NS: not significant. ** : $p < 0.005$.

## ii) Sex ratio of the $F_1$ generation produced by EST treated females

The number of males and females in the $F_1$ generation produced by 17 females treated by EST and 3 females from the control group, a total of 20, is given in Table 18. No difference was observed between the hatch ratio and the survival ratio after hatching in $F_1$ from these 20 pairs. All

6 samples in the 10 ppm and 50 ppm groups produced a 1:1 sex ratio of $F_1$. In the 100 ppm group, 7 out of 9 produced a 1:1 sex ratio. 2 in $F_1$ produced a sex ratio of 1:2.05 and 1:2.83 (female:male). One out of 2 in the 200 ppm group also produced a ratio of 1:3.04 in $F_1$. The sex ratio of $F_1$ of these 3 was 1:1 and was clearly significant ($P<0.005$). There was no significant difference between 1:3. All 3 from the control group produced 1:1 in $F_1$.

It has been reported that the $F_1$ sex ratio of 1:3 can be obtained for medaka (Yamamoto, 1958), gold fish (Yamamoto and Kajishima, 1968) and coho salmon (Hunter, 1982) by the mating of hetero sex chromosomes. Our results also agreed p. 22 with those in these reports. It was found that these 3 females were individuals whose sex differentiation was reversed from genetic males to functional females and that the sex chromosomes of rainbow trout are heterogamety.

4. Sex ratio of the $F_2$ generation of sex-reversed females and the viability of supermales (YY males)

The sex ratio of the $F_2$ generation was studied in order to elucidate the stucture of male sex chromosomes in the $F_1$ generation (1:3 female:male) produced by sex-reversed females (XY) and to confirm the viability of YY males.

1) Sample fish and methods

$F_1$ population with sex ratio of 18:51 (1:2.83), female:male obtained by mating sex-reversed females(XY)

appeared in the EST 100 ppm group with normal males(XY) was used for sample fish. 12 mature males and 9 mature females were mated with normal opposite sexes individually on the 2nd year and on the 3rd year respectively after hatching and the sex ratio of the $F_2$ generation was studied on the 15th month after hatching.

2) Results p. 23

No difference was observed in the hatching result or in the survival ratio up to the 15th month in 21 pairs of the $F_2$ generation obtained by samples of 12 males and 9 females. The number of appearances of males and females in the $F_2$ generation is given in Table 19.

9 $F_1$ males from No. 1 to No. 9 which were mated with normal females produced $F_2$ with a sex ratio of 1. 3 $F_1$ males from No. 10 to No. 12 produced all males in $F_2$. In the mating of $F_1$ females with normal males, all 9 pairs produced $F_2$ with a sex ratio of 1. 3 $F_1$ males which produced all males were YY males produced by mating XY females (sex- p. 24
reversed females) with XY males. It is thought that the production of all males(XY) was a result of mating with normal females(XX). It was found that the other 9 $F_1$ males were XY males and 9 $F_1$ females were XX females.

It was found that of 12 $F_1$ males, 9 were XY males and 3 YY males. The theoretical vlaue of XY male:YY male obtained by the mating of XY with XY is 2:1. No significant difference was observed between the experimental value

**Table 19.** Sex distribution of $F_2$ generation produced by mating males ($F_1$) or females ($F_1$) sired by sex reversed female, with normal females or males, respectively
F ; female M ; male

| Mating No. | | Sex distribution of $F_2$ | | Significance of difference of ratio from 1 : 1 |
|---|---|---|---|---|
| | | F | M | |
| $F_1$ male × normal female | 1 | 39 | 50 | NS |
| 〃 | 2 | 78 | 58 | NS |
| 〃 | 3 | 42 | 35 | NS |
| 〃 | 4 | 117 | 128 | NS |
| 〃 | 5 | 78 | 67 | NS |
| 〃 | 6 | 84 | 81 | NS |
| 〃 | 7 | 91 | 84 | NS |
| 〃 | 8 | 65 | 62 | NS |
| 〃 | 9 | 44 | 50 | NS |
| 〃 | 10 | 0 | 30 | ** |
| 〃 | 11 | 0 | 119 | ** |
| 〃 | 12 | 0 | 112 | ** |
| $F_1$ female × normal male | 1 | 13 | 18 | NS |
| 〃 | 2 | 32 | 36 | NS |
| 〃 | 3 | 19 | 16 | NS |
| 〃 | 4 | 31 | 31 | NS |
| 〃 | 5 | 10 | 11 | NS |
| 〃 | 6 | 23 | 22 | NS |
| 〃 | 7 | 13 | 10 | NS |
| 〃 | 8 | 10 | 17 | NS |
| 〃 | 9 | 16 | 24 | NS |

NS : not significant ** : $p < 0.005$

**Table 20.** Sex chromosome type of male mated with normal female and viability of YY male

| Sex chromosome | No. observed | No. expected (XY 2 : YY 1) | Significance of difference of No. expected |
|---|---|---|---|
| XY | 9 | 8 | NS |
| YY | 3 | 4 | NS |

NS : not significant

(XY 9:YY 3) and the theoretical value (XY 2:YY 1) (Table 20). It was assumed that YY males have the same viability as XY males. A YY male is shown in Plate III, 14.

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5. Maturation of all-male population of the $F_2$ generation and the sex ratio of the $F_3$ generation

The stages of maturation were observed in order to confirm that the sex chromosomes of all males obtained by mating YY males with normal females are XY and that all males are genetically normal males with normal reproductive functions. The sex ratio of the $F_3$ generation obtained by mating with normal females was also examined.

1) Sample fish and methods

2 groups of all-male populations produced by YY males were used for samples. On the 24th month from hatching, 25 fish from group No. 1 and 15 from No. 2 were collected randomly and the stages of maturation were examined. In order to observe the sex ratio of $F_3$, the milt from each fish from 5 mature males in No. 1 was fertilized. The tissue sections were made after 7 month and the sex ratio of $F_3$ was studied.

2) Results

The stages of reproduction of 2 all-male groups on the 24th month are given in Table 21. The ratio of mature males in this experiment was 28 % for No. 1 and 33 % for No. 2 and no difference was observed compared with standard values. The body weight had the trend that mature fish was heavier than immature fish, however, this trend is observed generally. No special change was observed in maturation of all males.

The fertilization ratio, the hatching ratio and the

**Table 21.** Ratio of mature males and gonadosomatic index (GSI) of 2 all-male groups produced by mating YY males with normal females at the stage of 24 months

| Group No. | No. observed | Mature male | Immature male | Ratio of mature male | Body weight ± SD | | GSI ± SD | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Mature | Immature | Mature | Immature |
| | | | | % | g | g | % | % |
| 1 | 25 | 7 | 18 | 28.0 | 70.9±28.2 | 64.8±34.4 | 3.31±4.1 | 0.17±0.2 |
| 2 | 15 | 5 | 10 | 33.3 | 73.9±17.8 | 63.9±39.2 | 2.56±1.00 | 0.08±0.05 |

GSI : gonad somatic index (gond weight/doby weight × 100)

**Table 22.** Ratio of hatched fry and sex distribution in $F_3$ produced by mating YY male's sons with XX females

| Mating No. | Ratio of fertilized egg | Ratio of hatched fry | Sex distribution | | Significance of difference of ratio from 1 : 1 |
|---|---|---|---|---|---|
| | | | F | M | |
| | % | % | | | |
| 1 | 93.1 | 78.2 | 6 | 4 | NS |
| 2 | 100.0 | 84.4 | 5 | 5 | NS |
| 3 | 100.0 | 81.6 | 5 | 5 | NS |
| 4 | 80.0 | 62.8 | 3 | 7 | NS |
| 5 | 100.0 | 88.4 | 5 | 5 | NS |

number of appearance of males and females of the $F_3$ generation produced by mating 5 males from NO. 1 with normal females are given in Table 22.

The fertilization ratio of 5 pairs was 80-100 %, mean 94.6 %, the hatching ratio 62.8-88.4 %, mean 79.1 % and high. No difference was observed between these ratios and the normal fertilization and hatching ratios. p. 25

The sex ratio of the $F_3$ generation was observed using tissue specimens. Females already had the oocytes in the perinucleolus stage and for males, the spermatogonia had formed the cyst membrane. Males and females were distinguished clearly. The sex ratio of $F_3$ for 5 pairs was 1 (Table 22).

It was confirmed from these results that the sex

chromosomes of all-male $F_2$ were XY and that no difference was observed from normal males in their maturation characteristics. No difference was observed between all males produced by YY males and normal males in genetics and in reproductive functions.

6. Discussion

The male gonad at hatch-out showed the same form as that of the female gonad. However, the increase in the spermatogonia was hardly observed at swim-up. Compared with females in which the tissues transfer to the meiosis at this time, a delay in the differentiation was observed. Further developments were also slow. A slow increase in the spermatogonia was observed from swim-up to the 8th week. It was on the 25th week that a testes formation became apparent through a formation of cyst membrane in the spermatogonia. However, an increase of the spermatogonia by the mitosis alone was observed and a transfer to the meiosis was not observed. The differences from the ovarian differentiation were apparent.

It has been observed commonly among many species of fish that the oogonia transfer to the meiosis much faster than the spermatogonia and that the latter continue to increase gradually for a long time. The same was observed in rainbow trout. Therefore the morphological change of reproductive cells cannot be used as a differentiation sign of testes.

Nakamura and Takahashi(1973) discussed the importance

of morphological change of the somatic element in the sex differentiation period in Tilapia. The same can be thought in the case of rainbow trout. In our observation, many somatic cells were observed in the male gonad at swim-up. This was clearly different from the female gonad. Therefore this can be thought to be a sign of testes differentiation, however, more detailed observations are necessary to clarify this point.

Feminization by sex steroids was studied using estrone and estradiol-17$\beta$ . Results from the 8 week-oral administration from swim-up showed that the reversal ratio was generally low and that the correlation between that and dosage levels was not evident. Okada(1973) reported a high reversal ratio by estrone administration in still-water breeding. It is thought that the differences of still-water breeding and running-water breeding and water temperatures might have been influential.

Nakamura(1981a) reported a high ratio of feminization in masu salmon. He stated that it could be achieved by an estrogen immersion treatment of hatched fry. In order to obtain a high ratio of feminization, a wide range of concentrations and administration from the preswim-up time should be studied.

From the results of mating sex-reversed females with normal males, the sex ratio of 1:3 female:male was obtained in $F_1$. Thus the functional feminization and the sex

chromosomes of male heterogamety were elucidated. From the masculinization experiment by MT administration, the sex chromosomes of female homogamety was also shown (Okada et al., 1979, Johnstone, 1979) and an XX-XY type was confirmed. Thorgaard(1977) reported an XX-XY type from the results of cytological observation. Our experimental results agreed with the results given in these reports.

YY males were confirmed in $F_1$ males produced by sex-reversed fish. Yamamoto(1975) reported that YY is lethal in white medaka of d-rR used in the experiment but viable in gold fish. He assumed that YY became lethal from the observation in which the functionless nodes of inactivated genes existed on Y chromosome and the active nodes with genes necessary for survival existed in the corresponding part on X chromosome. In gold fish, it was assumed that YY survived since no functionless nodes existed. From the results of mating XY-XY, Johnstone et al.(1979) assumed that YY could be lethal in rainbow trout. Hunter et al.(1982) assumed viability of YY in coho salmon. In this study, YY males were confirmed for the first time in salmonids and it was elucidated that there was no significant difference in viability between XY males and YY males. From this result, there is a strong possibility that the functionless nodes indicated by Yamamoto (1975) in medaka may not exist in rainbow trout. YY males had no abnormality in the classification characteristics such as the number of gill rakers and fin rays and matured normally.

It was confirmed that XY males sired by YY males matured normally and produced a 1:1 sex ratio in the next generation. It was elucidated that all males produced by using YY males were normal males genetically and functionally. It takes a long time to produce YY males. However, the production of all males using YY males is a safe and effective sex ratio control technique. p. 26

## Chapter IV Sterilization of breeding stock by artificially induced triploids

During the spawning period of cultured rainbow trout, problems related to maturation such as an increase in mortality, a suspension of growth and a decrease in the quality of meat are observed. When breeding is carried out beyond the maturation age, a decrease in the production efficiency due to these problems have been already indicated and this is a problem in the production of large-sized fish. It is thought that sterilization and suppression of maturation can eliminate problems related to maturation, improve the production efficiency of large-sized fish, make year round marketing possible and also simplify selection process. Therefore it is thought that sterilization and suppression of maturation are effective breeding techniques.

For the suppression of maturation, there are methods such as the adjustment of the breeding environment, the use of chemicals such as hormones and the use of $\gamma$-ray irradiation. However, complete sterilization is difficult with any of the

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above methods which have not been put to practical use.

It is known that sexual maturation becomes abnormal in the chromosome polyploids in plaice(Purdom, 1972), carp (Gervai et al., 1980), channel catfish(Walters et al., 1982) and Rhodeus ocellatus(Ueno and Arimoto, 1982). Allen and Stanley(1977) reported for river trout that sterilized fish which appeared in a hatchery were polyploids. Thorgaard (1979) made the same observation for rainbow trout and suggested the possibility of sterilization by polyploidy.

In order to study the sterilization of breeding stock by polyploidy, artificial triploids were produced by the hydrostatic pressure method used by Onosato(1983a) and maturation characteristics of triploids were elucidated. Methods of sterilization were also discussed.

1. Maturation characteristics of triploid rainbow trout

In order to elucidate the sexual maturation process of triploids, artificial triploids were produced and breeding tests were carried out. Mature fish which appeared in triploid males were mated with normal females and the characteristics of sperms produced by triploid males were observed.

1) Sample fish and methods

Within 15 minutes after normal fertilization, fertilized eggs were treated by hydrostatic pressure of 650 $kg/cm^2$ for 6 minutes and a release of the second polocytes of eggs was obstructed and triploids were produced. Polyploidy was

confirmed by making smear samples of blood cells by the standard method on the 37th month from hatching and by measuring the diameters of red blood cells and nuclei. Triploids were bred normally after hatching and appearances of mature fish were observed on the 15th month, 28th month and 37th month after hatching. Diploids from the same parent fish were used as control and were mixed with triploids and bred. The 2 groups were distinguished by marks on the lipid fins. On the 37th month, the gonad somatic indexes of mature fish and immature fish were compared. Mature males which appeared as triploids were mated with normal females and the developmental stages were observed. The morphology of sperms of diploids and triploids was observed under a scanning electron microscope(Alpha 10, Hitachi Seisakusho) after fixing the sperms with glutaraldehyde by the standard method. p. 27

## 2) Results p. 28

### i) Diameters of red blood cells

**Table 23.** Diameter of red blood cells and their nuclei of triploids (3n) and diploids (2n)

| Donor | No.of fish | Diameter of cell of RBC (N=420) | | Diameter of nucleus of RBC (N=200) | |
|---|---|---|---|---|---|
| | | Major diameter ±SD | Minor diameter ±SD | Major diameter ±SD | Minor diameter ±SD |
| | | μm | μm | μm | μm |
| 3n | 3 | 18.24±1.57 | 10.38±1.01 | 9.62±0.56 | 4.11±0.24 |
| 2n | 3 | 15.59±0.91 | 8.89±0.68 | 6.78±0.48 | 3.84±0.20 |

** : significant difference between triploid and diploid ($p<0.005$)

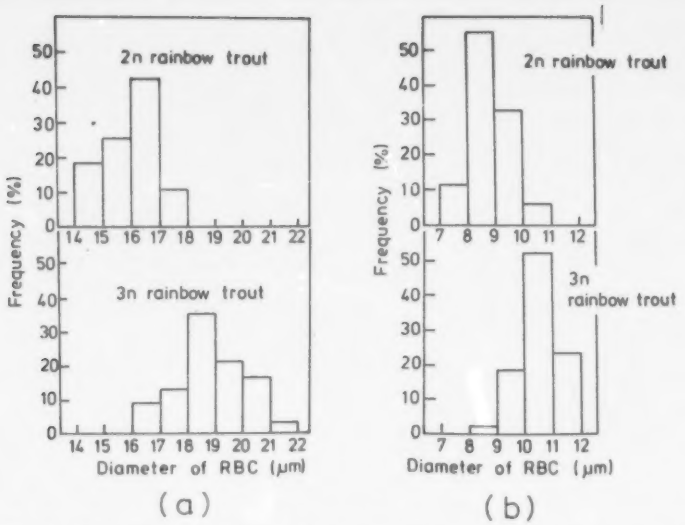


**Fig. 7.** Frequency distribution of major diameter (a) and minor diameter (b) of red blood cell (RBC) of diploids (2n) and triploids (3n). Four hundred and twenty RBC were measured each for diploids and triploids

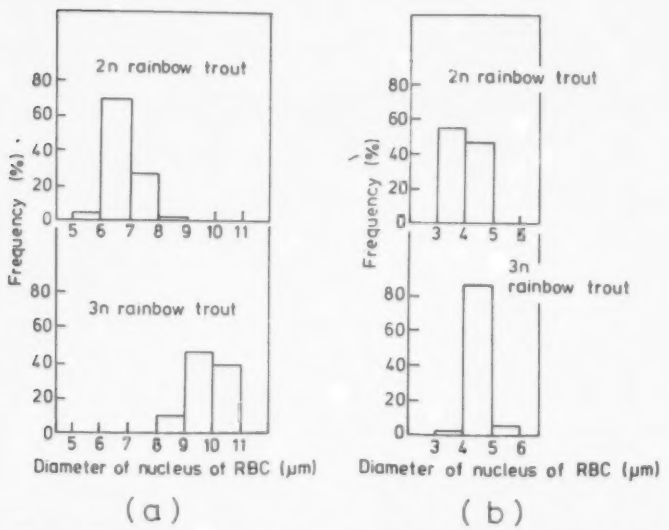


**Fig. 8.** Frequency distribution of major (a) and minor diameter (b) of red blood cell (RBC) of diploids (2n) and triploids (3n). Two hundred RBC were measured each for diploids and triploids

Diameters of red blood cells and nuclei of 3 triploids and 3 diploids were measured. Results are given in Table 23

and the histograms of measured values are given in Figs. 7 and 8. The long diameter of the red blood cell was 18.24 $\mu$m for triploids and 15.59 $\mu$m for diploids. The short diameter was 10.38 $\mu$m for triploids and 8.89 $\mu$m for diploids. Both diameters of triploids were 1.17 times those of diploids and the difference was clearly significant ($P<0.005$). The long diameter of the nucleus of triploids was 1.41 times that of diploids. The short diameter of triploids was 1.1 times that of diploids. A tendency was observed that the diameter became larger in the direction of long axis according to the degree of polyploidy. For long and short diameters, the difference between triploids and diploids was significant ($P<0.005$). A phenomenon in which the shape of the red blood cell changes to a larger size due to the polyploidy of chromosomes is known for gold fish (Sezaki et al., 1977, Onosato et al., 1983) and for Atlantic salmon (Benfey and Sutterlin, 1984b). It has been indicated that the measured results of diameters of the red blood cells and the degree of polyploidy have a high correlation. In this experiment, the increase ratio in long and short axes of the red blood cell differed from the above results, however, significant differences in the diameters of blood cells and nuclei of triploids and diploids were observed. It was confirmed that polyploidy in rainbow trout can be easily distinguished also by measuring the red blood cells. The red blood cells of triploid and diploid are given in Plate III, 15 and Plate

**Table 24.** Number of mature fish and body length of triploids (3n) and diploids (2n) at various ages after hatch out
F; female, M; male

| Month old | 3n rainbow trout | | | | 2n rainbow trout | | | |
|---|---|---|---|---|---|---|---|---|
| | No. of fish | | | BL±SD | No. of fish | | | BL±SD |
| | immature | mature F | mature M | | immature | mature F | mature M | |
| mo. | | | | cm | | | | cm |
| 15 | 58 | 0 | 0 | 10.2±2.0 | 20 | 0 | 0 | 9.2±2.4 |
| 28 | 41 | 0 | 6 | 24.3±2.0 | 12 | 0 | 3 | 23.7±3.7 |
| 37 | 19 | 0 | 18 | 28.7±3.1 | 5 | 4 | 6 | 26.8±3.5 |

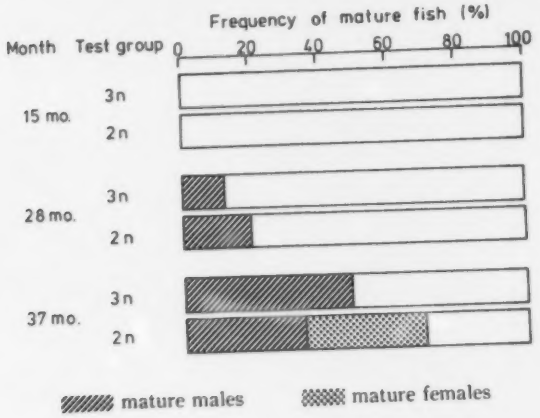


**Fig. 9.** Frequency of mature fish of triploids (3n) and diploid (2n) at various age (mo.)

III, 16 respectively.

ii) Maturation stages of triploids

Maturation stages and body lengths in the spawning period are given in Table 24 and the appearance ratio of mature fish is given in Fig. 9. No mature fish were observed in diploids and triploids on the 15th month of the spawning period. On the 28th month, 3 mature males(20 %) in diploids and 6 (12.8 %) in triploids appeared. On the 37th month, 4 mature females and 6 mature males were observed in diploids and 66 % of all diploids matured. In triploids, mature fish

**Table 25.** Body weight (BW), fork length (FL), condition factor (CF) and GSI of dipioids (2n) and triploids (3n) at 37 months after hatch out

| Maturity | N | BW | FL | CF | GSI |
|---|---|---|---|---|---|
| | | g | cm | | % |
| Mature male | 2n(6) | 415.1±132.4 | 32.1±3.4 | 12.185±0.546 | 2.47±0.85* |
| | 3n(5) | 382.3±52.7 | 31.5±1.7 | 12.192±0.335 | 1.33±0.48 |
| Mature female | 2n(6) | 404.8±106.0 | 31.8±3.0 | 12.344±0.589 | 13.82±2.75 |
| | 3n(0) | | | | |
| Immature male | 2n(1) | 205.6 | 26.7 | 10.802 | 0.03 |
| | 3n(0) | | | | |
| Immature female | 2n(5) | 226.3±70.2** | 27.1±2.1** | 11.124±1.546 | 0.25±0.08** |
| | 3n(4) | 379.8±65.5 | 32.7±2.0 | 10.829±0.178 | 0.04±0.02 |

*: significant difference between 2n and 3n ($0.02 < p < 0.05$)
**: significant difference between 2n and 3n ($p < 0.005$)
CF: body weight/fork length$^3$ × 1000.

were 18 males only as no mature females appeared. 48.6 % of all triploids matured. Measured results of mature and immature fish on the 37th month are given in Table 25. The sex ratio of triploids was 1:1, the same as that of diploids. It was assumed that the sex chromosomes of triploids were XXX female and XXY male. Judging from the sex ratio of 1:1 and the maturation ratio of about 50 %, it is thought that most males matured by the 37th month. GSI for triploids is 1.33 % and smaller than that for diploids which is 2.47 % ($0.02 < P < 0.05$).

Some abnormalities such as the storing of a large p. 29
quantity of serous fluid in the spermiduct are observed. Sperms are released by pressing the abdominal area (Plate III, 17; IV, 18). The seminal fluid which was squeezed out was clearly thinner than that of diploids. The tissue images

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of a diploid and a triploid are shown in Plate IV, 19 and 20. No remarkable difference was observed in the structures of testes of diploids and triploids. The testes of triploids were filled with sperm. However, a difference was observed between a diploid and a triploid in the scanning electron microscope images (Plate IV, 21 and 22). The sperm heads of the triploid are somewhat larger than those of the diploid and angular in shape. Some changes in the size are also observed.

GSI for immature females was 0.25 % for diploids and 0.04 % for triploids and a significant difference was observed. In the immature ovary of a diploid (Plate IV, 23), the granules could be confirmed with the naked eye. However, in the ovary of a triploid (Plate V, 24), they were small and difficult to distinguish from the ovary even under a stereoscopic microscope. The tissue images of ovaries of a diploid and a triploid are shown in Plate V, 25 and 26. The oocytes of the diploid are in the late perinucleolus stage to the yolk vesicular stage. Most of the oocytes of the triploid are in the karyosomes in the synapsis stage and the oocytes in the perinucleolus stage are very rare. Many images of the regression of the reproductive cells and the connective tissue were observed. The tissue image of the ovary of female triploids demonstrated similar characteristics to the ovaries of triploids for plaice (Purdom, 1972) and for channel catfish (Wolters, 1982).

For maturation of triploids, developmental abnormalities of gonads have been reported in Salverinus fontinalis (Allen and Stanley, 1977), Salmo gairdneri (Thorgaard and Gall, 1979), Pleuronectes platessa (Purdom, 1972) and Ictalurus punctatus (Wolters et al., 1982). The same abnormalities shown in these species of fish were also observed in the results of this experiment. Furthermore, in the 3 year-old fish, the oocytes of females stayed at the fry stage and a remarkable difference from those of diploids was observed. Therefore female triploids can be thought to be sterile.

For the growths of diploids and triploids, no difference was observed in the body lengths, body weights and weight gains in mature males. However, significantly larger triploids were obtained for immature females.

iii) Mating results of mature triploid males with normal females

Triploid males matured mostly by the 37th month. A portion of them was mated with normal females. The seminal fluid of males was thinner than that of diploids but had inseminating ability. However, a delay in the hatching time was observed and the hatchability decreased considerably. No hatched fry showed a normal external form. Considerable abnormalities such as curved vertebrae, short bodies and abnormal eyeballs were observed in all individuals. There has been only one example of mating mature triploid males with normal females for pleuronectes platessa (Lincoln, 1981). p. 30

According to this report, the hatchability was extremely low and hatched fry died within 48 hours. The same results were obtained for rainbow trout. It is thought that the decrease in the hatchability and the appearance of abnormalities were caused by the following. In the meiosis of a triploid, a disproportion occurred in the pairing of chromosomes and heteroploid sperms were produced. Eggs were inseminated by heteroploid sperms and developmental abnormalities appeared. It is thought that the abnormal forms of sperms observed under a scanning electron microscope were caused by heteroploidy.

2. Production and growth of all-female triploids

In triploid rainbow trout, females were completely immature even in the 3rd year. Judging from the developmental stages of the oocytes, it was thought that females were immature for life. In males, mature fish appeared in the 2nd year and most fish matured by the 3rd year. Even though triploids can be produced, the practical effectiveness of their sterility as broodstock is very small.

Effectiveness of sterilization can be achieved by producing all-female triploids using sex-reversed XX males and by breeding them as broodstock. In order to study production methods of all-female triploids using sex-reversed males, the appearance ratio of triploids produced by hydrostatic pressure and their growth characteristics were observed.

**Table 26.** Growth, hepato-somatic index (HSI) and gonadal development of triploid and diploid females at 15 months after hatching

| Polyploidy | N | BW ± SD | BL ± SD | HSI ± SD | Gonadal development | |
|---|---|---|---|---|---|---|
| | | | | | undifferentiated | Female |
| | | g | cm | % | | |
| Triploid | 129 | 14.5 ± 8.5** | 9.8 ± 1.9** | 1.31 ± 0.41 | 129 | 0 |
| Diploid | 21 | 23.5 ± 13.5 | 11.4 ± 2.31 | 1.23 ± 0.35 | 0 | 21 |

**: significant difference between triploid and diploid ($p < 0.001$)
HSI: hepato somatic index (liver weight/body weight × 100).

1) Sample fish and methods

All-female triploids were produced by mating normal females with sex-reversed XX males and by applying hydrostatic pressure (Onosato, 1983). Fertilization was done by the standard process. Within 15 minutes of fertilization, 650 kg/cm$^2$ of hydrostatic pressure was applied for 6 minutes. Hatched fry were bred normally. On the 15th month, 150 fish were taken and the diameters of red blood cells were measured and the degree of polyploidy was determined. Then the appearance ratio of triploids was obtained. Measurements were taken of body lengths and body weights of diploids and triploids whose polyploidy became apparent. Growth characteristics of the 2 groups were compared.

2) Results

i) The appearance ratio of triploid fish and the development of gonads

The ratio of eyed-eggs of sample fish was 69.6 % and the hatching ratio was 52.5 %, somewhat low. The cause of the reduction in the hatching ratio cannot be determined.

However, there is a possibility of the inclusion of bad eggs since the time of the experiment was at the end of the spawning period. Polyploidy was determined by the diameters of red blood cells described in this study (triploid 18.24 x 10.38 $\mu$m, diploid 15.59 x 8.89 $\mu$m, Table 23) as a standard. Results showed that 129 fish (86.0 %) were triploids and 21 (14.0 %) were diploids (Table 26).

It is known that the appearance ratio of artificially produced triploids is very high. When a low temperature treatment was applied immediately after fertilization, 95 % of hatch-out fish for Rhodeus ocellatus (Ueno and Arimoto, 1982) and 100 % of normal hatch-out fish for carp (Gervai et at., 1980) and for plaice (Purdom, 1972) became triploids. Compared with these results, the appearance ratio of triploids in this experiment was low. The cause of a decrease in the appearance ratio is unknown. It is also unknown whether these diploids were normal diploids or gynogenetic diploids developed by a disappearance of the male pronucleus for some reason.

Gonads of all 129 triploids showed a thread-like external appearance. Many oocytes at the synapsis stage in the early meiosis were observed in the tissue sections. Ovaries were not yet developed. Gonads of diploids could be distinguished clearly from the ovaries. Normally developed oocytes at the perinucleolus stage were observed. From these results, it was confirmed that the sex chromosomes of

p. 31

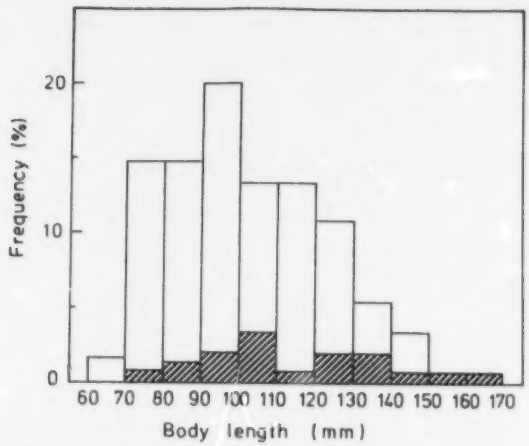


**Fig. 10.** Frequency distribution of body length of hydrostatic pressure treated rainbow trout at 15 months after hatch out. Shaded area represents diploids unexpectedly appeared among the same group. Mean body lenbth of triploids was 9.8±1.9 (SD) cm. and diploids was 11.4±2.31 cm. Difference was significant between triploids ($P<0.001$)

triploids were of XXX type of all females and that the individuals developed as diploids were of XX type of all females.

9 (7.0 %) out of 129 triploids had the spleen separated into two parts (Plate V, 27). This phenomenon was not observed in diploids and was noted as a morphological change associated with polyploidy. No difference was observed in the hepatosomatic indices(HSI) of diploids and triploids (Table 26).

ii) Growth of diploids and triploids

Body lengths and body weights on the 15th month are given in Table 26 and a histogram of body lengths in Fig. 10.

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Diploids were larger than triploids by 9 g in body weight and by 1.6 cm in body length. The difference was clearly significant ($P<0.001$).

On the growth difference between diploids and triploids, there have been reports that no difference was observed at the immature stage for three-spined stickleback (Swarup, 1959), plaice (Purdom, 1972), carp (Gervai et al., 1980) and channel catfish (Wolters et al., 1982). Results of the present experiment disagree with those in the above reports. Diploids were the individuals which did not become triploids by hydrostatic pressure treatment. Their composition of chromosomes (genome composition) could not be determined. Therefore it is questionable whether the results of our experiment can be regarded as general characteristics of triploid rainbow trout. Gonads of triploid females showed the thread-like external appearance. In the tissue observation, many oocytes in the karyosome stage were seen and a close resemblance was noted with the 37 month-old ovaries described in the previous section. In diploid females, egg granules were observed externally. Many eggs of the late perinucleolus stage were observed. They were clearly different from those of triploid females. From these observations, it is thought that triploid females grow sterile and diploid females mature normally. Therefore it is assumed that 86 % of the sample fish are sterilized and 14 % mature as females.

3. Discussion

Artificially induced triploids were bred for 37 months after hatching. Results showed that most males matured completely but that no mature female fish were observed. Normal females mature in 3 years. The ovaries of triploid females were very undeveloped and the reproductive cells remained at the same stage as that at the time of swim-up. It is judged that they do not reach maturity even if breeding is continued further. Many oocytes were at the karyosome stage. Very few oocytes at the early perinucleolus stage were observed in later stages of the development. Many images of regression of the oocytes were also observed. Therefore it is thought that the reproductive cells develop until the karyosome stage and then they regress after that stage. The oocytes at the karyosome stage move to the meiosis stage and then enter the growth stage. However, in triploids, the oocytes do not grow but regress due to the heteroploidy of the chromosomes. This phenomenon is very interesting from the viewpoint of the development of the oocytes and the structure of the genomes.

In triploid males, abnormalities such as a decrease in GSI and abundant serous components were observed. However, they showed secondary sexual characteristics similar to diploids and matured. In mating with normal eggs, an extreme decrease in the hatching ratio and the appearance of abnormal fish were observed. It was assumed from the unequal meiosis

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that the sperms were heteroploidy.

Lincoln(1981) reported a decrease in the hatching ratio and the abnormality of hatch-out alevin from his study of plaice in which normal eggs were inseminated by triploid male sperm. The same results were obtained from our study. It is thought that the abnormality of triploid sperm is common to these species of fish.

In the growths of triploids and diploids, there were no difference among three-spined sticleback (Swarup, 1959), plaice (Purdom, 1976), Rhodeus ocellatus (Ueno and Arimoto, 1982) and carp (Gervai et al., 1980). It was reported that for channel catfish (Wolters et al., 1982), no growth difference was observed in the immature stage but the growth of triploids was better than that of diploids after maturation. It can be said that essentially there is no growth difference between diploids and triploids among these species. In our experiment, the immature triploids of 37th months showed better growth than that of immature diploids in the test for maturation characteristics. However, in the test of all-female triploids, diploids showed a better result. It is hoped that further study can elucidate this point.

14 % of diploids appeared in the polyploidy induced by hydrostatic pressure. It was not clear whether these diploids were individuals (normal 2n) produced by a failure to prevent the release of second polocyte or those (gynogenesis 2n) of polyploidy induced by the addition of pressure

after the male pronuclei disappeared without karyogamy. The appearance ratio of polyploids by hydrostatic pressure and the developmental mechanism of diploids which exist among triploids should be elucidated in future. When XX males were used in producing polyploids, 86 % were triploid females which were sterile and 14 % were diploid females which matured. In future, 100 % sterilization can be achieved by improving the probability of polyploidy treatment.

From this study, it became apparent that triploid females were sterilized almost invariably. It was demonstrated that the sterilization of broodstock is realizable by producing all-female triploids using sex-reversed XX males. Using this treatment, the difficulty with respect to maturation in the production of large-sized rainbow trout can be removed and production cost will be reduced. Year round shipments and energy saving in the selection operation can be realized and as a result production efficiency will be improved.

## Chapter V Summary

Salmonids have been utilized for a long time as an important fishery resource. Many studies have been done and their physiology and ecology are becoming known in detail. Especially in the reproduction process, techniques of artificial fertilization and artificial hatching have been established and they are being managed artificially. In rainbow trout which are an important cultured species, the process

of stock production to parent fish is under artificial management. Complete cultivation is carried out in many regions.

The purpose of this study was to establish a cultivation technique which will improve the production efficiency of rainbow trout cultivation. Considering various advantages and disadvantages caused by sex differences, the control of sex ratio in stocks which is suitable for cultivation and production of each sex is very useful in improving production efficiency. Maturation of cultivated fish is a large factor in the decrease in cultivation efficiency which aims at the production of fish meat. It is expected that the sterilization of stocks will remove the problem due to maturation and improve production efficiency greatly.

Control of the sex ratio in stocks was discussed in the study of masculinization of genetic females and feminization of genetic males. For the control of sex ratio, a method was established in which all females or all males could be produced in the $F_1$ or $F_2$ generation from sex-reversed fish using sex steroids. A production method for all females is given in Fig. 11. It is a method in which genetic females are masculinized and all females are obtained by mating these sex-reversed males with mormal females. A production method for all males is given in Fig. 12. It is a method in which genetic males are feminized, YY males are confirmed in $F_1$ obtained by mating with normal males and all males are obtained

by mating YY males with normal females. It is not a direct method of obtaining the intended sex ratio by administration of sex steroids. It is an indirect method in which the sex chromosomes of sex-reversed fish and the paradox in apparent sexes are utilized and the sex ratio is controlled by mating with the normal opposite sex. Method of utilizing directly p. 34
the sex-reversed generation is easy. However, sex reversals are not always complete and various gonadal abnormalities at various stages, including hermaphrodites, appear. Therefore the sex control is incomplete. Furthermore when fish are reproduced using sex-reversed fish, the sex ratio of $F_1$ produced is abnormal and also YY males are produced which effects the sex ratio of progenies. It also remains questionable whether the hormone-treated generation should be used for food. The indirect method is to produce individuals which produce either X sperms or Y sperms using sex reversal and to produce all females or all males by mating with normal females. It requires time and labor but 100 % sex control is achieved and all females and all males produced are genetically and functionally normal. The indirect method is excellent for practical use. In this method, the sex is controlled by using the sperms, therefore it is possible to control the sex of a large quantity of eggs by keeping only a few XX males and YY males.

XX males, XY females and YY males produced by sex reversal do not exist in the natural world. When they are

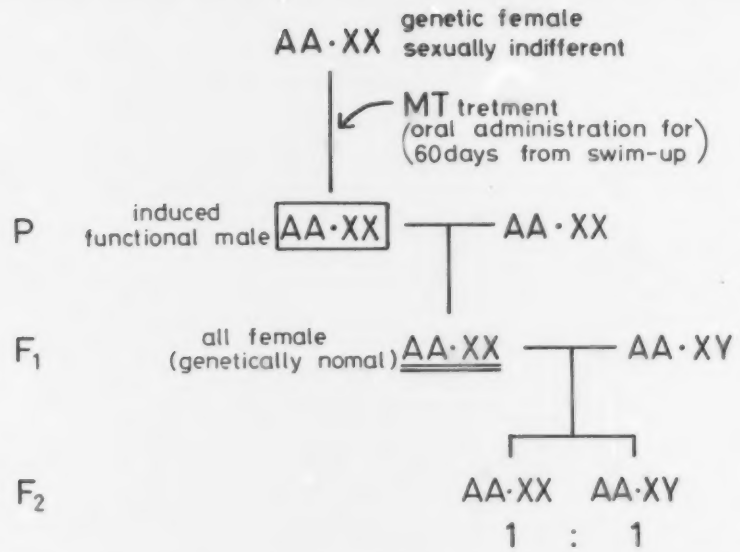


**Fig. 11.** Method for producing all-female brood of rainbow trout. AA ; autosome, XX ; sex chromosome type of female, XY ; sex chromosome type of male

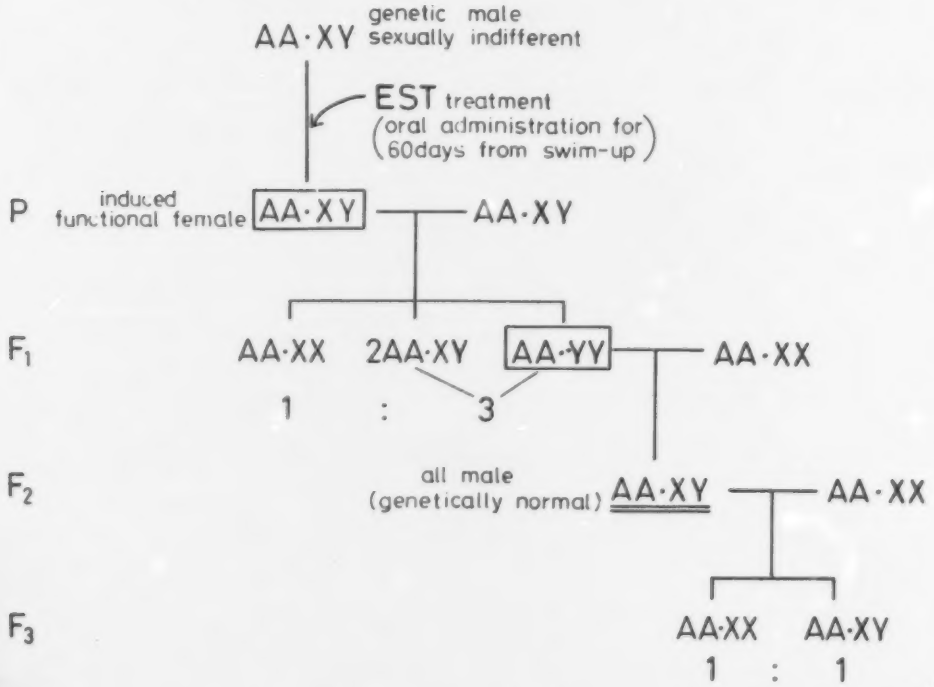


**Fig. 12.** Method for producing all-male brood of rainbow trout. AA ; [illegible]ome, XX ; sex chromosome type of female, XY ; sex chromosome type of male

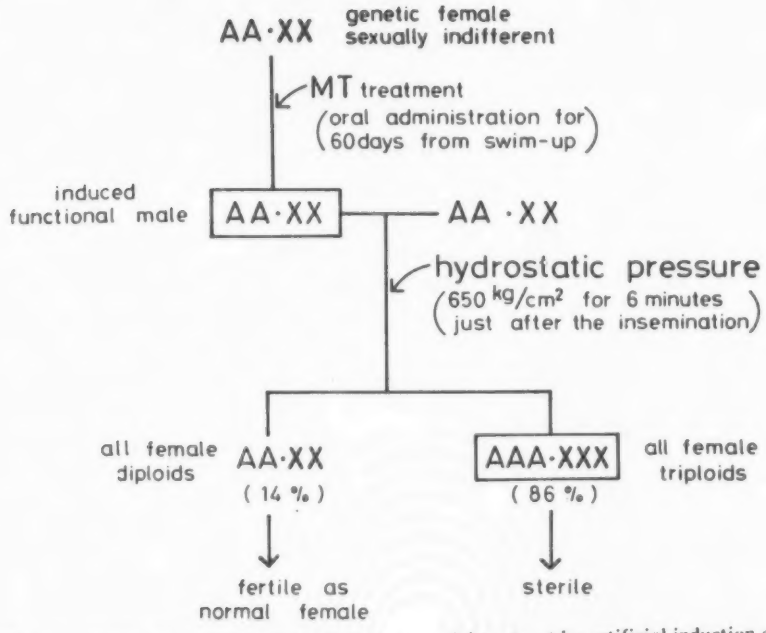


**Fig. 13.** Method for producing sterile brood of rainbow trout by artificial induction of all female triploids. AA, autosome XX, sex chromosome type of female

used in reproduction, the sex ratio of 1:1 changes greatly as stated in this report. It is expected that when these individuals are released to the natural world, the sex ratio in their progenies will change. Therefore the sex-reversed fish should be kept under artificial management and used only for the control of the sex ratio of broodstocks.

In rainbow trout, the production of all-female stocks by XX males established by this study is beginning to be put to practical use. A technique for controlling the sex ratio which was established for cultivation of rainbow trout is also beginning to be applied to cultivation of other species of salmonids. Control of the sex ratio by the same method

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as for rainbow trout is being established in coho salmon, young masu salmon and Atlantic salmon. It is expected that it is possible to control the sex ratio in the same manner in the cultivation of dog samon, masu salmon and pink salmon. Control of the sex ratio in cultivated fish should be studied carefully and based on many experimental results considering that their growing ground is the natural world and the natural sex ratio of male to female is 1:1.

In order to remove difficulties due to sexual p. 35
maturation, the sterilization of broodstock was discussed in the study of sterilization of broodstock by artificially induced triploidy. It was confirmed from the observation of the maturation stages of artificially induced triploidy that triploid males matured but triploid females were sterile for life. A high ratio of triploid females was produced by applying hydrostatic pressure immediately after fertilization using XX males. It was confirmed that these triploid females were sterile. In this study, it was estimated that 86 % were sterilized as triploid females and 14 % matured as diploid females. The fact that sterilization of more than 80 % was achieved suggests that this is very useful sterilization technique. This also improves the production efficiency of large-sized fish which are required to be bred beyond their maturation age (Fig. 13). It is necessary to study further the economical efficiency of triploid females. Causes for the appearance of diploid females which were not polyploids

by hydrostatic pressure treatment should be elucidated.

In order to improve the production efficiency of broodstocks of rainbow trout, artificial control of the sex ratio in broodstocks and sterilization of stocks were studied and the establishment of techniques was attempted. These techniques are not limited to rainbow trout but also can be applied to other species of salmonids and contribute to progress in propagation and cultivation of salmon.

Summation of all chapters

Summary of our study is given below.

(1) From the results of the observation of the process of sex differentiation of genetic females, it was confirmed that a portion of the oogonia shift to the meiosis at swim-up and it became clear that the differentiation towards females had already begun.

(2) The oocytes increase by the 4th week after swim-up and occupy a large portion of the ovary and the differentiation to female is completed by this time.

(3) From the result of the study of the duration of 17α-methyltestosterone administration which is effective in masculinization, a high ratio of reversal of sex differentiation was demonstrated by starting the treatment at swim-up or within 2 weeks of swim-up and by continuing for 8 weeks.

(4) From the results of the study of the dosage levels of 17α-methyltestosterone(MT) and 11-ketotestosterone (11KT) which are effective in masculinization, a high ratio

of reversal of sex differentiation was demonstrated by oral administration of 0.5-1 ppm of MT or about 5 ppm of 11KT for 8 weeks from swim-up.

(5) An all-female $F_1$ was obtained by mating sex-reversed males with normal females. It was confirmed that the sex chromosomes of rainbow trout were female homogamety.

(6) It was confirmed that an all-female $F_1$ produced by sex-reversed fish consisted of genetically and functionally normal females. It was demonstrated that all females produced by XX males have no problems in utilization.

(7) From the results of the observation of the process of sex differentiation of genetic males, the transition of the reproductive cells to the meiosis observed in females at swim-up was not observed. The reproductive cells increased gradually and an increase of somatic cells was observed.

(8) Transition of the spermatogonia to the meiosis was not observed even on the 25th week from swim-up. However, a form as testes is completed at this time with development of the seminiferous tubule and formation of cyst membrane of the spermatogonia.

(9) From the results of the study of the dosage level of Estradiol-17$\beta$ which is effective in feminization, feminization was observed at 50 ppm and 100 ppm with low ratio. It is necessary to conduct a detailed study of dosage levels and duration of administration in order to obtain efficient feminization.

(10) A sex ratio of 1:3 (Female:male) in $F_1$ was obtained by mating females sex-reversed by Estrone with normal males. It was confirmed that the sex chromosomes of rainbow trout were male heterogamety.

(11) YY males which were assumed to exist in $F_1$ males were confirmed. It was demonstrated that all-male $F_2$ are produced by mating YY males with normal females.

(12) It was confirmed that all-male $F_2$ which was produced by YY males consisted of genetically and functionally normal males. It was demonstrated that all males produced by YY males have no problems in utilization. p. 36

(13) In order to observe the maturation stages of triploid rainbow trout, artificial triploids were bred up to the 37th month from swim-up.

(14) By the 3rd year, triploid males showed secondary sexual characteristics clearly and most of them matured. However, females did not mature. It was clear that they were sterilized with the ovary at the immature stage of karyosome.

(15) Abnormalities such as a reduction of GSI and an excess of serous fluid were observed in triploid males. However, they produced sperms which were capable of fertilization. No normal fry were observed from the results of fertilization. It was assumed that these sperm were heteroploidy.

(16) Results from the production of all-female

triploids using sex-reversed XX males showed that 86 % were triploid females and 14 % were diploid females.

(17) Clear results could not be obtained with regard to the growth difference between diploids and triploids.

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## Plate I

Plate I-1 to -4 are transverse sections

1 Gonad in genetic female at hatching. Oogonium (og) is visible in the gonad. sc ; somatic cells.
Bar represents 10 $\mu$m.

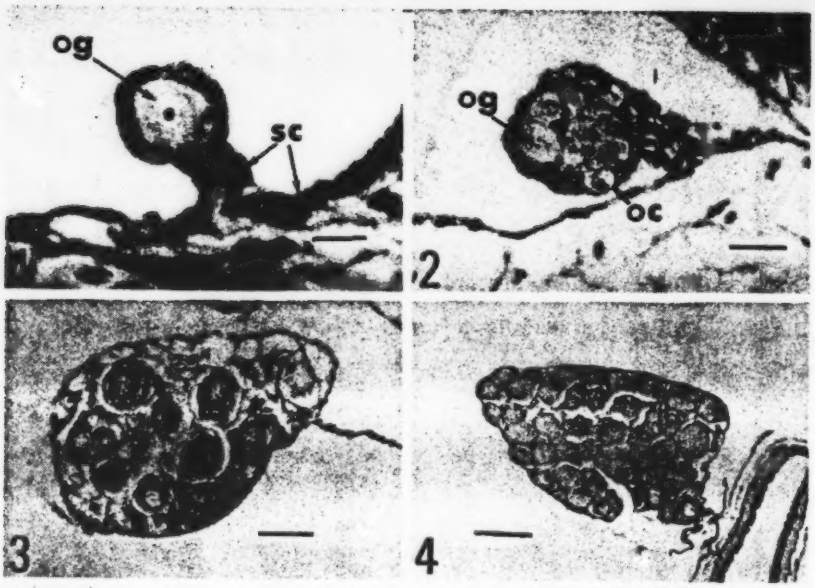
2 Gonad in genetic female at the swim-up stage. Oogonia (og) increase in number. Oocyte (oc) in observed occasionally.
Bar represents 20 $\mu$m.

3 Gonad in genetic female 6 weeks after swim-up. Oocytes are in early meiosis.
Bar represents 30 $\mu$m.

4 Gonad 8 weeks after swim-up. Oocytes are in the early peri-nucleolus stage. bv ; blood vessels.
Bar represents 50 $\mu$m.

5 Normal testes (ts) of induced male by 1 ppm methyltestosterone. Milt can be squeezed out.

6 Compressed testes (ts) lacking in the genital duct of induced male by 1 ppm methyltestosterone. Milt cannot be squeezed out.



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## Plate II

Plate II-10 to -12 are transverse secions.

7 Unilateral tentis (ts) of induced male by 1 ppm methyltestosterone. The left testis is compressed and the right is thread-like. Sperm duct is not observed.

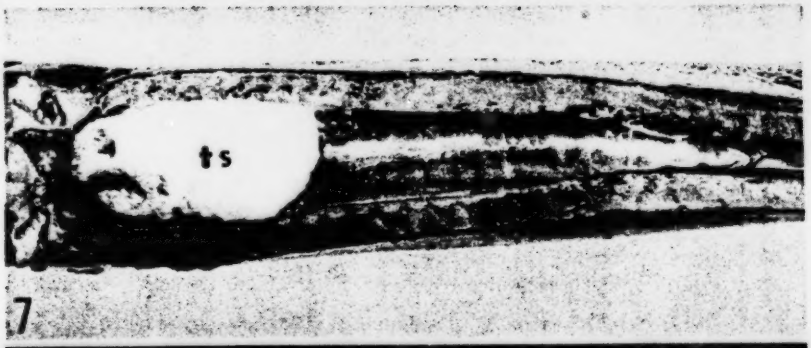
8 Hermaphroditic gonad observed in 1 ppm methyltestosterone-treated fish. The left gonad in hermaphroditic and the right is ovary.

9 A XX male induced 1 ppm methyltestosterone treatment.
Bar represents 5 cm.

10 Gonad of a genetic male at the swim-up stage. sg: spermatogonia, sc; somatic cells.
Bar represents 10 $\mu$m.

11 Gonad of a genetic male 8 weeks after the swim-up stage. Spermatogonia increase in number. bv; blood vessels, sd; sperm duct.
Bar represents 20 $\mu$m.

12 Testis of male 25 weeks after the swim-up stage. bv; blood vessels, sd; sperm duct.
Bar represents 70 $\mu$m.

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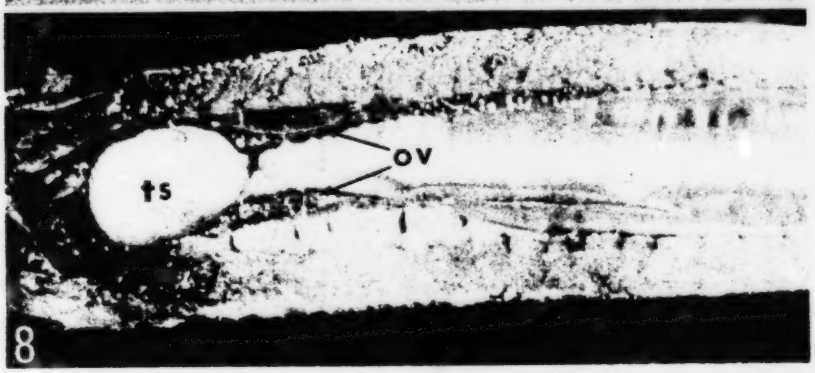


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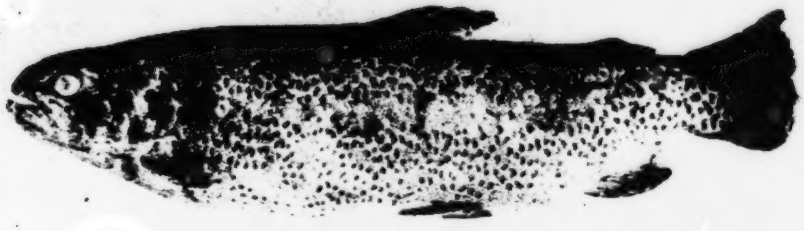

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## Plate III

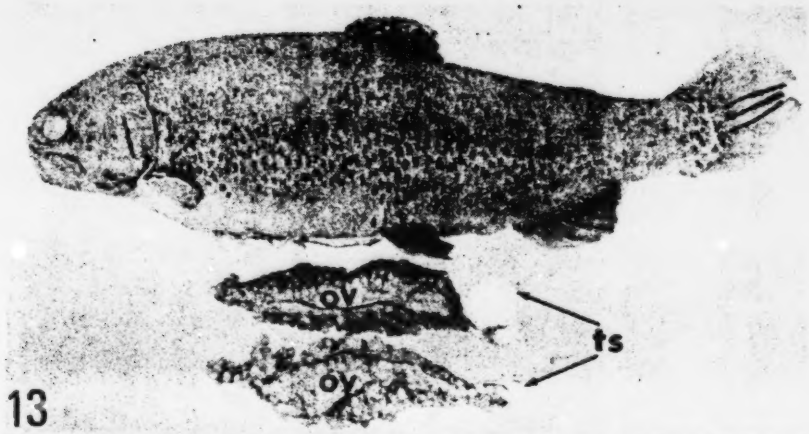
A hermaphrodite appearing in 50 ppm estrone-treated fish (above) and it's hermaphroditic gonads (blow). ov; ovary, ts; testis. Bar represents 5 cm.

14 A YY male (above) with mature testes (ts, below) produced by mating XY female with XY male.
Bar represents 5 cm.

15 Erythrocytes of triploid fish.
Bar represents 10 $\mu$m.

16 Erthrocytes of diploid fish.
Bar represents 10 $\mu$m.

17 Testes (ts) of mature triploid fish with abnormal sperm duct (sd).
Bar represents 1 cm.

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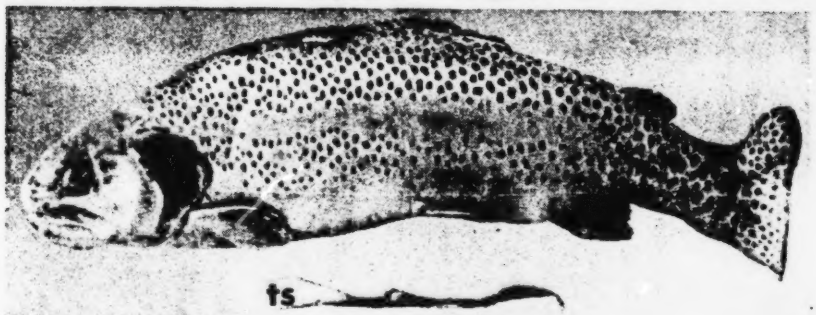


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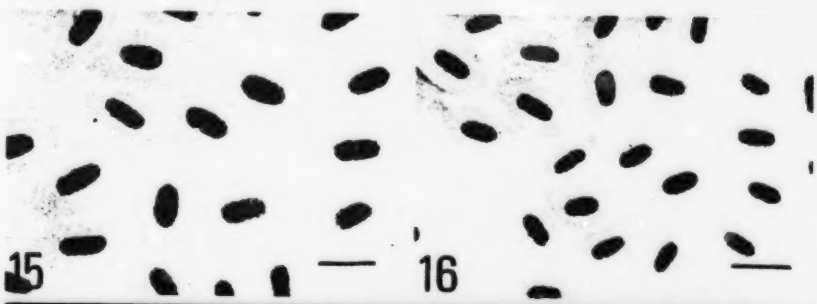

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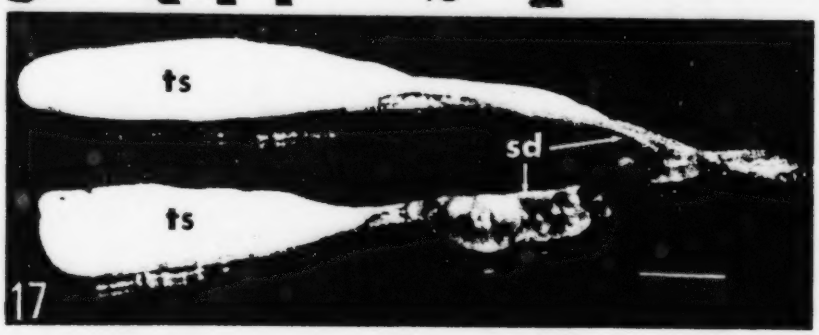


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## Plate IV

18 Testis (ts) of mature triploid fish. lv; liver

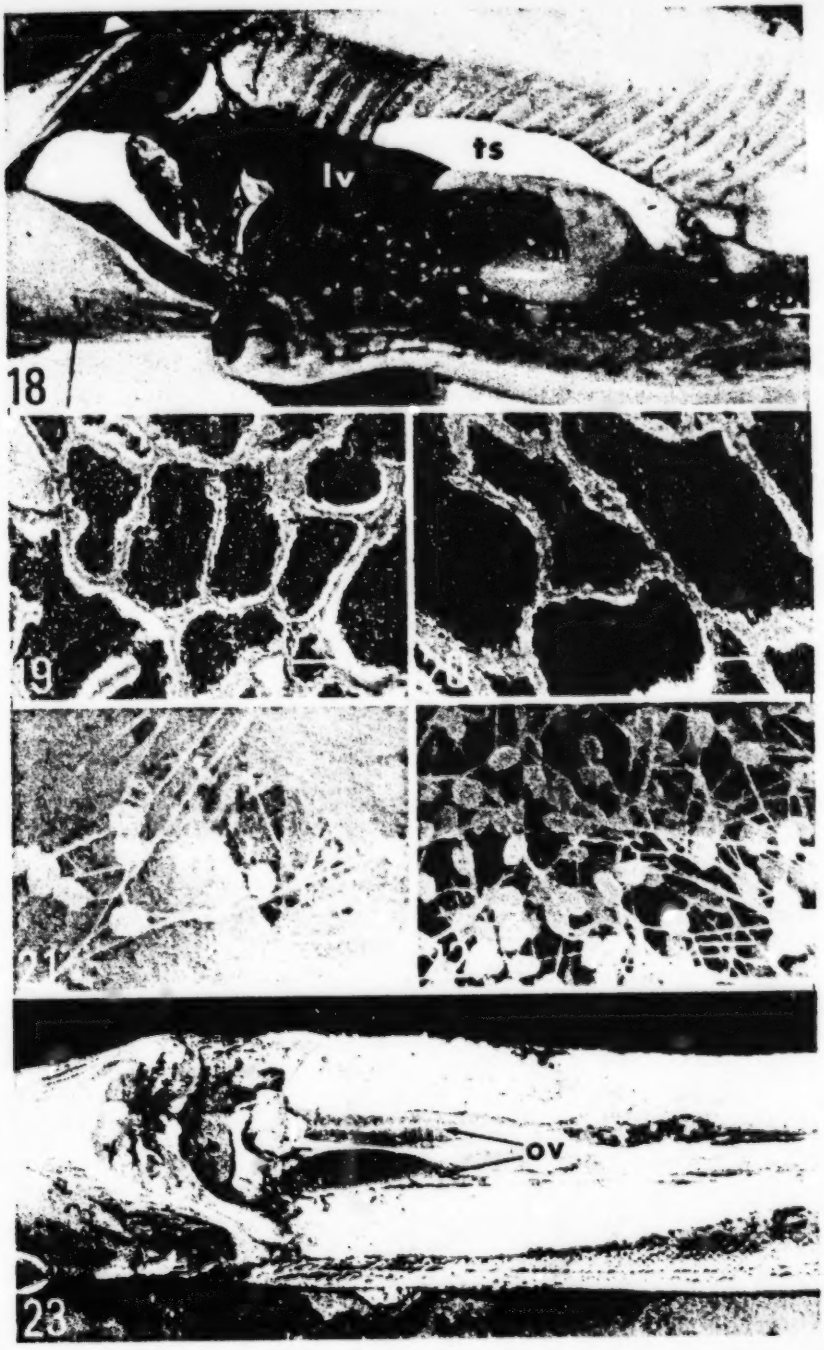
19 Transverse section of mature testis of diploid fish.
Bar represents 10 $\mu$m.

20 Transverse section of mature testis of triploid fish.
Bar represents 10 $\mu$m.

21 Scanning electron microgmaph of sperm of triploid fish.
Bar represents 2 $\mu$m.

22 Scanning electron micrograph of sperm of diploid fish.
Bar represents 2 $\mu$m.

23 Immature ovary (ov) of diploid fish.


lv
ts
18
9
ov
23

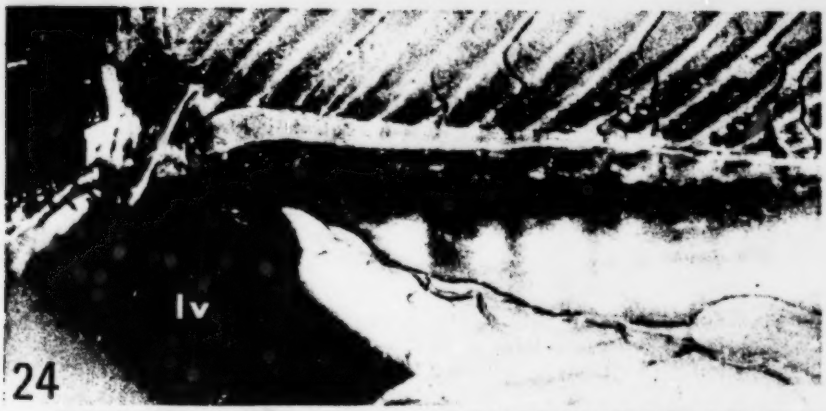
## Plate V

24 Undeveloped ovary (ov) of triploid fish. lv ; liver.

25 Transverse section of ovary of diploid fish.
Bar represents 10 $\mu$m.

26 Transverse section of ovary of triploid fish. oc ; oocyte in the late peri-nucleolus stage. Arrows show undeveloped germ cells.
Bar represents 10 $\mu$m.

27 Abnormal spleen (sp) observed in triploid fish.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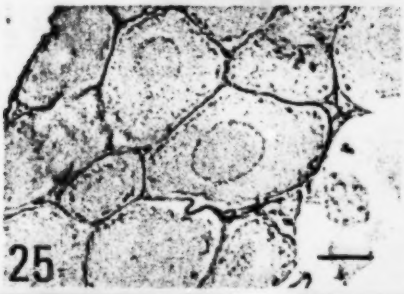
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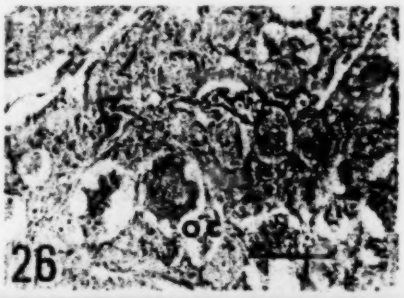
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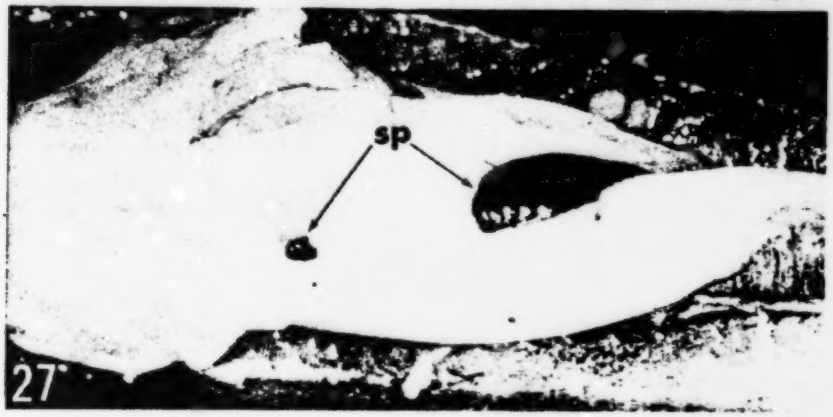
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